Identification of the Polypyrimidine Tract Binding Protein-associated Splicing Factor-p54(nrb) Complex as a Candidate DNA Double-strand Break Rejoining Factor*

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The biological effects of ionizing radiation are attributable, in large part, to induction of DNA double-strand breaks. We report here the identification of a new protein factor that reconstitutes efficient double-strand break rejoining when it is added to a reaction containing the five other polypeptides known to participate in the human nonhomologous end-joining pathway. The factor is a stable heteromeric complex of polypyrimidine tract-binding protein-associated splicing factor (PSF) and a 54-kDa nuclear RNA-binding protein (p54(nrb)). These polypeptides, to which a variety of functions have previously been attributed, share extensive homology, including tandem RNA recognition motif domains. The PSF-p54(nrb) complex cooperates with Ku protein to form a functional preligation complex with substrate DNA. Based on structural comparison with related proteins, we propose a model where the four RNA recognition motif domains in the heteromeric PSF-p54(nrb) complex cooperate to align separate DNA molecules.

Living organisms are exposed to ionizing radiation from many sources. Biological effects of ionizing radiation include cell death, mutation, and transformation. The principal radiation target is DNA, and the most potent DNA lesions are double-strand breaks (DSBs), caused when an ionization track creates clustered damage that affects both strands (1). DSBs disrupt the physical integrity of the chromosome. If not repaired prior to cell division, they are often fatal to the cell. Also, incorrect joining of DSBs creates translocations and other chromosomal aberrations, leading to genetic instability, oncogene activation, and cancer.

A main pathway of DSB repair in mammals is nonhomologous end joining (NHEJ). At least five polypeptides participate in mammalian NHEJ (reviewed in Refs. 2 and 3). They include the two subunits of Ku, which bind to DNA ends; DNA ligase IV (L4) and XRCC4 (X4), which form a complex that catalyzes strand ligation; and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which regulates the reaction. It is likely that additional NHEJ factors remain to be discovered. The five known polypeptides are insufficient to reconstitute regulated, high efficiency DNA end joining in a cell-free reaction (4). The addition of a small amount of nuclear extract to such a reaction, however, greatly increases its efficiency, suggesting the presence of additional DSB repair factors in the extract. The factors may accelerate the reaction by aligning separate DNA ends; such activity is likely to be crucial in vivo to prevent diffusion and reassortment of ends when multiple DSBs occur simultaneously. Although Ku and DNA-PKcs can hold separate DNA molecules together in vitro, such complexes are only marginally stable (5–8), suggesting the involvement of additional proteins.

To identify the repair factors present in nuclear extracts, we established a functional assay in which biochemical fractions, derived from HeLa cell nuclear extracts, were tested for their ability to stimulate end joining in the presence of recombinant Ku and L4-X4. In previous work, we identified two different stimulatory fractions (4, 9). One fraction contains a >500-kDa complex of human Mre11, Rad50, and NBS1 polypeptides. These proteins, and their Saccharomyces cerevisiae homologs, have previously been implicated in DSB repair (10–13). The other fraction contains a ~200-kDa factor that does not cross-react with antibodies against any of a variety of candidate proteins previously implicated in DSB repair. Only the latter fraction is capable of cooperating with DNA-PKcs to establish a phosphorylation-regulated end joining reaction (9). The two factors appear to participate in alternative, parallel pathways of DNA ligase IV-dependent end joining.

We report here the identification of the ~200-kDa factor as a complex of polypyrimidine tract binding protein-associated splicing factor (PSF) and p54 nuclear RNA-binding protein (p54(nrb)). These related polypeptides each contain tandem RNA recognition motifs (RRMs), together with conserved, homologous flanking sequences (14, 15). Previous studies have suggested multiple functions for the PSF-p54(nrb) complex, including DNA recombination and RNA synthesis, processing, and transport (Refs. 16–19; reviewed in Ref. 20). We show here that the PSF-p54(nrb) complex strongly stimulates DNA end joining in vitro, binds directly to the DNA substrates of the end joining reaction, and cooperates with Ku to establish a functional preligation complex.

EXPERIMENTAL PROCEDURES

DNA End Joining Assays—DNA end joining assays contained recombinant L4-X4 complex (21) and non-His-tagged Ku heterodimer (22). Some reactions also contained DNA-PKcs, which was purified using an

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† The abbreviations used are: DSB, double-strand break; PSF, protein-associated splicing factor; RRM, RNA recognition motif; NHEJ, nonhomologous end joining; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; mAb, monoclonal antibody; hnRNP, heterogeneous nuclear ribonucleoprotein.
affinity column containing 5 mg of a C-terminal Ku80 peptide (KGS-
GEEDGVDDLLDMI) (23). The column was equilibrated with buffer A
(25 mM HEPES-KOH (pH 7.5), 10% glycerol, 1 mM dithiothreitol, 0.1
mM EDTA) containing 0.05 mM KCl. Nuclear extracts from 12.5 liters of
HeLa cell culture (4) were passed over the column, which was eluted
with a 0.05 to 1 mM KCl gradient in buffer A. DNA-PKcs-containing
fractions were further purified using a 1-ml Mono S ion exchange column
pre-equilibrated with buffer DB (0.1 mM KOAc, 20 mM Tris-HCl (pH 7.9),
1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) and protease inhibitors
(10 μg/ml phenylmethylsulfonyl fluoride and 1 μg/ml each of pepstatin A,
soybean trypsin inhibitor, leupeptin, and aprotinin). The column was eluted
with a 0.1 to 0.5 mM KOAc gradient in buffer DB.

End joining reactions were performed in a volume of 20 μl and
contained 50 mM triethanolamine-HCl, 10 mM Tris-HCl (pH 7.9), 85
mM KOAc, 0.25 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 1.0
mM Mg(OAc)2, 100 ng/ml bovine serum albumin, 1 mM ATP, 0.5 μg/ml
substrate DNA (BamHI-linearized pUC19 plasmid, 5' end-labeled
with polyethylene glycol kinase and [γ-32P]ATP), and proteins as indi-
cated in the figure legends. The reactions were assembled without
DNA and preincubated for 5 min at 37 °C. DNA was added, and
incubation was continued for 30 min at 37 °C. The products were
analyzed as described (4).

**Purification of DNA End Joining Stimulatory Factors**—The factors
were purified using nuclear extract from 50 liters of HeLa cell culture.
Heparin-agarose, Q-Sepharose (0.3 mM KOAc eluate), and Superdex 200
chromatography were performed as described (9). Active fractions from
the Superdex 200 column were pooled and loaded onto a 10-ml single-
strand DNA-agarose column (GE Healthcare, Piscataway, NJ), and eluted
with a linear gradient of 0.1 to 1 mM KOAc in Buffer DB. The active
differences in each fraction. Spots of interest were ex-
cised and subjected to in-gel tryptic digestion. The peptides were
analyzed by matrix-assisted laser desorption and ionization (MALDI)
mass spectrometry.

**Immunoprecipitation**—Coupled in vitro transcription-translation re-
actions were performed using the TNT in vitro transcription/translation
kit (Promega, Madison, WI). Metabolic labeling was performed as de-
scribed previously (24). Immunoprecipitation was performed as de-
scribed (24) in IPP buffer (10 mM Tris (pH 7.4), 0.5 M NaCl, and 0.1%
Nonidet P-40). For immunodepletion, protein A-Sepharose beads (GE
Healthcare) were loaded with antibody (specified in the figure legends)
onight at 4 °C. The beads were washed with IPP buffer and then
with Buffer DB. Q-Sepharose 0.3 mM KOAc eluate was added (30 μl),
and incubation was continued for 2 h. Supernatant was used for end joining
assays.

**Electrophoretic Mobility Shift Assays**—The reactions (10 μl) contained
buffer DB, DNA substrate as in end joining assays, and PSF:p54(nrb) and
Ku as indicated in the figure legends. Protein-DNA complexes were
resolved by native PAGE and visualized by PhosphorImager analysis.

**RESULTS**

**Purification and Characterization of a Novel End Joining Factor**—We have described previously a biochemical complementation
assay for the identification of NHEJ factors (9). Two
active fractions were obtained, one of which contains a ~200-kDa factor that is not antigenically cross-reactive with any of a
number of candidate proteins (9). Further purification of this
factor was performed (Fig. 1A). Activity resolved as a single
peak on single-strand DNA-agarose (not shown) and Mono S
columns (Fig. 1B). SDS-PAGE analysis showed two major
polypeptides with mobility corresponding to ~55 and 100 kDa
that co-eluted with peak stimulatory activity (Fig. 1C).

We examined the dependence of factor activity on the other
NHEJ proteins in the reaction. Consistent with our previous
results, very little activity was seen with Ku and L4-X4 alone
under the conditions used (9). In the presence of the factor,
DNA end joining activity was absolutely dependent on L4-X4 (Fig. 1D, compare lanes 2 and 5), excluding the possibility that the factor itself has DNA ligase activity. End joining was also substantially dependent on Ku (compare lanes 3 and 5). Quantification of these results showed that activity in the presence of Ku and the stimulatory factor together was ~5-fold greater than the sum of the activity in reactions with either protein alone (lane 5 compared with sum of lanes 3 and 4). This synergy suggests that Ku and the new factor have different functions in the end joining reaction.

DNA-PKcs was not required for end joining in the reconstituted system. Rather, the addition of DNA-PKcs consistently led to a modest inhibition of activity (Fig. 1E, compare lanes 3, 5, and 7). The addition of DNA-PKcs also sensitized the reactions to LY294002, a small molecule inhibitor of DNA-PKcs and other phosphatidylinositol 3-kinase family members (25). In the absence of DNA-PKcs, LY294002 had no effect (Fig. 1E, lane 4), whereas in the presence of 2.4 and 3.6 nM DNA-PKcs, it decreased activity by 2.0- and 4.6-fold, respectively (lanes 6 and 7). The results are consistent with previous findings using partially purified factor (9). They are also consistent with a model (26) where DNA-PKcs regulates the end joining reaction by binding to and blocking the free DNA ends and is then released by autophosphorylation. Inhibition by DNA-PKcs in the absence of LY294002 suggests that the intrinsic efficiency of this release is less than 100%. However, the addition of the inhibitor, together with DNA-PKcs, clearly led to a further decline in end joining activity.

Identification of the Factor as a Heteromeric Complex of PSF and p54(nrb)—The polypeptides in the active fractions were analyzed by two-dimensional difference gel electrophoresis (“Experimental Procedures”). Two major spots (labeled A and B in Fig. 2A, left panel) are seen in the active fraction 17. These correspond in molecular mass to the major polypeptides in the one-dimensional SDS-PAGE analysis (Fig. 1C). The two-dimensional difference gel electrophoresis method allows comparison of two samples, labeled with different fluorophores, run in the same gel (27). The comparator sample here was an inactive trailing fraction from the Mono S column, fraction 24. Spots A and B were markedly less abundant in this fraction than in the peak fraction (Fig. 2A, compare left and right panels). A three-dimensional rendering of this region of the gel makes the magnitude of the difference clear (Fig. 2B).

Polypeptide A migrated as a basic protein of 100 kDa. The three-dimensional rendering (Fig. 2B) shows that it migrates as a single peak, with some material trailing to the basic side. Polypeptide B migrated as a basic protein of 55 kDa. It runs as twin peaks, both of which were later shown to contain the same tryptic peptides.

Spots corresponding to polypeptides A and B were excised and digested extensively with trypsin, and the products were analyzed by mass spectrometry. Good peptide coverage was obtained for both spots (Fig. 2C). Comparison of the tryptic fingerprint patterns with the nonredundant protein data base identified Spot A as polyprimidime binding PSF (SwissProt accession number P23246) and Spot B as p54(nrb) (SwissProt accession number Q15233). Although PSF has a predicted molecular mass of 74 kDa, it has previously been shown to migrate at a position corresponding to 100 kDa in SDS-PAGE (16). The observed mobilities of the two polypeptides are otherwise consistent with the prediction based on the sequence.

Conserved sequence motifs identified in PSF and p54(nrb) are diagrammed in Fig. 2C. The central and C-terminal regions of the two polypeptides are related with 59% identity and 72% similarity overall. Both polypeptides contain RRM domains, which are also present in a variety of other RNA and single-strand DNA-binding proteins (reviewed in Ref. 28). The RRMs are embedded within a longer Drosophila behavior, human splicing motif shared by these and several other proteins (15). A region of similarity between PSF and p54(nrb), not shared by other family members, extends beyond this motif to the C termini. Predicted coiled-coil regions, within and adjacent to the Drosophila behavior, human splicing motif, coincide with a region previously shown to mediate dimerization (29).

Immunodepletion of PSF-p54(nrb)—To characterize antibodies for use in an immunodepletion experiment, we immunoprecipitated PSF and p54(nrb) produced by in vitro transcription and translation of cDNA clones (Fig. 3, A and B) or by [35S]methionine metabolic labeling in human cells (Fig. 3C). When PSF and p54(nrb) were translated separately in vitro, monoclonal antibody (mAb) targeted against each polypeptide precipitated only that polypeptide, with no detectable cross-reactivity (Fig. 3B, lanes 1, 2, 4, and 5). When PSF and p54(nrb) were co-translated, antibody against each polypeptide co-precipitated the other (lanes 3 and 6). Neither polypeptide was precipitated by control, nonspecific mouse IgG1 (lanes 7–9). We note also that two smaller products were visible in the p54(nrb) in vitro translation and co-immunoprecipitation experiments (Fig. 3, A, lanes 2 and 3, and B, lanes 3, 5, and 6). These presumably arise from premature termination or the use of alternative initiation codons in the p54(nrb) gene and have not been investigated further.

When cell lysates containing endogenous PSF-p54(nrb) were used in an immunoprecipitation reaction, anti-PSF mAb co-
precipitated a polypeptide corresponding in size to p54(nrb) (Fig. 3C, lane 2), and anti-p54(nrb) mAb co-precipitated a polypeptide corresponding in size to PSF (lane 3). The identity of the co-precipitated polypeptides was confirmed in separate experiments by immunoblotting (data not shown). These results confirm a previous report that PSF and p54(nrb) form a stable complex in vivo and in vitro (29).

Immunodepletion experiments were performed using these antibodies to confirm that the PSF/p54(nrb) complex was the active component in the end-joining factor preparation. The PSF/p54(nrb) complex was partially purified to separate it from the other stimulatory activity (the Mre11-Rad50-NBS1-associated factor) that is present in crude cell extracts (4). This preparation, corresponding to the Q-Sepharose 0.3 M KOAc eluate (see Fig. 1A), was subjected to immunodepletion with anti-PSF mAb or anti-p54(nrb) mAb. Immunodepletion reduced end-joining activity to background (Fig. 3D, lanes 2 and 8). Control immunodepletion with nonimmune mouse IgG1 had no effect (lane 4). The addition of highly purified PSF/p54(nrb) restored full activity to the depleted extracts (lanes 7 and 9) but had little effect on control extracts (lanes 3 and 5). The results demonstrate that stimulatory activity is either intrinsic to the PSF/p54(nrb) complex or resides in a tightly associated protein.

**PSF/p54(nrb) Binding Substrate DNA**—The DNA binding properties of PSF/p54(nrb) were characterized in an electrophoretic mobility shift assay using the same DNA as in the end joining assays (Fig. 4A). The addition of increasing amounts of protein resulted in progressively more slowly migrating complexes (lanes 1–4). Ku alone formed a different complex with DNA (lane 5). The addition of a small amount of Ku with PSF/p54(nrb) resulted in formation of distinctive, slowly migrating complexes that differed in mobility from those formed with either protein alone (lanes 6–8). The results were similar whether Ku was held constant and PSF/p54(nrb) concentration varied or vice versa (lanes 9–16). The results indicate that Ku and PSF/p54(nrb) are capable of binding DNA both separately and together.

To determine whether complexes formed by Ku, PSF/p54(nrb), and DNA were functional intermediates in the pathway toward ligation, we preincubated combinations of separately purified Ku and PSF/p54(nrb) with either of two substrates, which differed in length but had compatible ends (Fig. 4B). We determined whether preincubation of PSF/p54(nrb) resulted in “commitment” to ligate that substrate in preference to a second substrate. Characteristic ladders of ligation products were seen with each substrate (lanes 2 and 4). As expected, little activity was seen in reactions where PSF/p54(nrb) or Ku were omitted (lanes 1, 3, 5, and 6). Lanes 7–12 show the substrate commitment experiment. The key result is seen by comparing lanes 10 and 11, where a DNA substrate that was preincubated with PSF/p54(nrb) complex and Ku, together, was ligated in preference to another fragment that was preincubated with Ku alone. Thus, preincubation with PSF/p54(nrb) and Ku resulted in formation of a committed, preligation complex. A control reaction was performed where both substrates were preincubated, separately, with PSF/p54(nrb) and Ku, then mixed, and assayed (lane 12). A mixed ladder of products was seen. A similar result was obtained when the two DNA substrates were mixed at the outset (lane 13).
We present three lines of evidence that establish the PSF-p54(nrb) complex as a candidate DNA end-joining factor: (a) PSF and p54 (nrb) are principal components of an end-joining stimulatory fraction, which was purified solely on the basis of its activity with no prior assumptions about its active constituents. (b) Absorption of a crude stimulatory fraction with monoclonal antibodies to PSF and p54 (nrb) depleted end joining activity. Activity was restored by the addition of purified PSF-p54(nrb) complex. (c) The PSF-p54(nrb) complex cooperated with the other proteins known to participate in NHEJ in vivo. Activity was dependent on Ku and L4-X4 and was regulated by DNA-PKcs phosphorylation. PSF-p54(nrb) also formed a committed preligation complex with Ku and DNA substrate.

Previous work has identified multiple functions for the PSF-p54(nrb) complex in the cell nucleus. It binds U5 small nuclear RNA and may participate in splicing (29). It also retains hyperedited heterogenous nuclear RNAs and unspliced lentiviral RNAs in the nucleus (17, 18), and it modulates nuclear receptor activity (16). We propose that the PSF-p54(nrb) complex has an additional, previously unrecognized function in NHEJ. Precedent for dual function in RNA biogenesis and NHEJ is provided by Ku protein itself, which associates with transcribed regions of chromatin in vivo and influences transcription by both direct (30) and indirect (31) mechanisms. There are also examples of proteins with dual functions in transcription and base or nucleotide excision repair (32–35).

As previously shown by our laboratory and others, isolated L4-X4 is highly active on homopolymeric model substrates in the absence of stimulatory factors (21, 36). In comparison, activity on natural double-strand DNA fragments is much lower, particularly under conditions used here where DNA substrate concentration is low (0.5 ng/μl). Presumably, the function of both the PSF-p54(nrb) and the Mre11-Rad50-NBS1 stimulatory factors is to increase the effective concentration of DNA ends by stabilizing pairing in a preligation complex. NHEJ enables mammalian cells to withstand doses of radiation (in the range of 1–4 Gray) that induce dozens of breaks simultaneously. Although repair begins within minutes, it does not reach completion for several hours (37). Establishment of a preligation complex may stabilize the genome during this crucial interval.

A model for how PSF-p54(nrb) might stabilize paired DNA ends is provided by the structure of another tandem RRMs-containing protein, hnRNP A1, in a complex with single-strand

**Fig. 5. Tandem RRMs promote DNA pairing.** A, binding of hnRNP A1 RRMs to single-strand telomere DNA, based on crystal structure (38). Protein binds as a dimer; DNA strands are anti-parallel and cross perpendicular to the protein dimer interface, such that each strand contacts one RRM in each protein monomer. B, hnRNP A1 RRM domains promote pairing of DNA segments. Topology of paired DNAs is one of several that have been proposed based on crystal structure (38). C, model for preligation complex. PSF-p54(nrb) binds to free end of substrate DNA via paired RRM domains (one in each subunit). Formation of a region of single-strand DNA near the end may facilitate binding. Two of four RRM domains in the PSF-p54(nrb) complex remain unoccupied and potentially stabilize pairing with a second DNA substrate. Coiled-coil interactions between subunits (not shown) may further stabilize the PSF-p54(nrb) complex.

**Fig. 4. Binding of PSF-p54(nrb) to DNA.** A, electrophoretic mobility shift assay. Binding reactions contained PSF-p54(nrb) and Ku as indicated with 0.05 nM linearized plasmid DNA, B, design of commitment assays. Preincubation mixes contained Ku (0 or 8 nM), purified PSF-p54(nrb) (0 or 6 nM), and one of two DNA substrates (0.5 nM). After 10 min at 37 °C, the preincubation mixes were in some cases combined. Purified L4-X4 (16 nM), ATP, and other reaction constituents were added; incubation was continued; and the products were analyzed by 0.6% SDS-agarose gel electrophoresis. C, results of commitment assays. Lanes 1–6, single-substrate reactions. Lanes 7–12, two-substrate reactions. Indicated preincubation mixes were combined prior to the addition of L4-X4 and ATP. Lane 13, two-substrate control reaction. All of the components were mixed prior to the start of preincubation.

Reactions were also performed where one substrate was preincubated with PSF-p54(nrb) alone and the other with Ku alone. Preincubation with PSF-p54(nrb) alone resulted in substrate commitment (lanes 8 and 9), but total activity was much lower than in lanes 10 and 11. This indicates the PSF-p54(nrb) and Ku cooperate only when present in cis on the same DNA; they do not cooperate in trans.

**DISCUSSION**

We present three lines of evidence that establish the PSF-p54(nrb) complex as a candidate DNA end-joining factor: (a) PSF and p54 (nrb) are principal components of an end-joining stimulatory fraction, which was purified solely on the basis of its activity with no prior assumptions about its active constituents. (b) Absorption of a crude stimulatory fraction with monoclonal antibodies to PSF and p54 (nrb) depleted end joining activity. Activity was restored by the addition of purified PSF-p54(nrb) complex. (c) The PSF-p54(nrb) complex cooperated with the other proteins known to participate in NHEJ in vivo. Activity was dependent on Ku and L4-X4 and was regulated by DNA-PKcs phosphorylation. PSF-p54(nrb) also formed...
telomeric DNA (38). In this co-crystal structure, tandem RRM domains of two hnRNP A1 monomers are paired in a side-by-side, rotationally symmetric arrangement (Fig. 5A). Two anti-parallel segments of single-strand DNA cross the protein complex perpendicular to the protein dimer interface. Thus, the hnRNP A1 dimer facilitates pairing of DNA segments (Fig. 5B, 2) styled after Ref. 38).

The four RRM domains of the PSF:p54(nrb) complex potentially bind DNA in a similar way. In general, RRM domains bind preferentially to single-strand nucleic acids. In the model shown in Fig. 5C, we have drawn PSF:p54(nrb) as binding to an induced single-strand segment proximal to the DNA ends, with Ku having translocated to an internal position. We have also drawn PSF:p54(nrb) as binding in trans to two DNAs simultaneously. Although the model shown is consistent with available data, we note that the detailed geometry of the Ku:PSF:p54(nrb):DNA complex has yet to be investigated experimentally.

A cell or animal model lacking PSF or p54(nrb) function has yet to be characterized. In preliminary experiments, small interference RNA-mediated knock-down of p54(nrb) in human somatic cells was incompatible with long term survival in a clonogenic assay, which precluded the use of such assays to measure radiation sensitivity.2 The apparent loss of cell viability may arise because of the multiple functions of PSF (9, 10). Nevertheless, it may be feasible to characterize the effect of loss of p54(nrb) and PSF function on shorter term surrogate endpoints of radiation injury.

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Identification of the Polypyrimidine Tract Binding Protein-associated Splicing Factor–p54(nrb) Complex as a Candidate DNA Double-strand Break Rejoining Factor

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