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Characterization of DNA binding and pairing activities associated with the native SFPQ•NONO DNA repair protein complex

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Abstract

Nonhomologous end joining (NHEJ) is a major pathway for repair of DNA double-strand breaks. We have previously shown that a complex of SFPQ (PSF) and NONO (p54^{nrb}) cooperates with Ku protein at an early step of NHEJ, forming a committed preligation complex and stimulating end-joining activity by 10-fold or more. SFPQ and NONO show no resemblance to other repair factors, and their mechanism of action is uncertain. Here, we use an optimized microwell-based assay to characterize the *in vitro* DNA binding behavior of the native SFPQ•NONO complex purified from human (HeLa) cells. SFPQ•NONO and Ku protein bind independently to DNA, with little evidence of cooperativity and only slight mutual interference at high concentration. Whereas Ku protein requires free DNA ends for binding, SFPQ•NONO does not. Both Ku and SFPQ•NONO have pairing activity, as measured by the ability of DNA-bound protein to capture a second DNA fragment in a microwell-based assay. Additionally, SFPQ•NONO stimulates DNA-dependent protein kinase autophosphorylation, consistent with the ability to promote formation of a synaptic complex formation without occluding the DNA termini proper. These findings suggest that SFPQ•NONO promotes end joining by binding to internal DNA sequences and cooperating with other repair proteins to stabilize a synaptic pre-ligation complex.

Keywords

DNA repair; DNA binding; nonhomologous end joining; SFPQ; NONO

1. Introduction

Nonhomologous end joining (NHEJ) is the default pathway for DNA double-strand break repair in mammalian cells. In classical, or canonical, NHEJ, Ku protein recognizes broken

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DNA ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and other DSB repair factors (reviewed in [1–3]). Previously, we observed that purified core NHEJ factors were insufficient for DNA end joining *in vitro*, and that activity was greatly stimulated by a heterodimer of SFPQ (also known as polypyrimidine tract binding protein-associated splicing factor, or PSF) and NONO (also known as 54 kDa nuclear RNA binding protein, or p54^{nrb}) [4, 5]. Both SFPQ and NONO have since been found to participate in the DNA damage response *in vivo* [6–10]. They also have independent functions in RNA biogenesis and the regulation of gene expression (for example, [11–13], reviewed in [14, 15].

SFPQ and NONO are part of a small family of tandem RNA recognition motif-containing proteins, with no evident sequence similarity to other known repair factors, and their mechanism of action is hence not clearly understood. Here we describe use of a novel binding assay to characterize DNA interaction and pairing activities of native SFPQ•NONO isolated from cultured mammalian cells. Data are consistent with a model where SFPQ•NONO and Ku protein interact with substrate DNA simultaneously at non-overlapping sites, stabilizing assembly of a repair complex.

2. Materials and Methods

2.1 DNA substrates

A 2686 bp plasmid (pUC19, Invitrogen, Carlsbad, CA) was linearized with HindIII. Following phenol:CHCl₃ extraction and EtOH precipitation, DNA was incubated with 40 μM biotin-7-dATP, 100 μM each of dTTP, dCTP, and dGTP, 50 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 100 μM dithiothreitol, 50 μg/ml bovine serum albumin, and 2 Units of Klenow fragment DNA polymerase at 37 °C for 1 h. The reaction was heated to 70°C for 20 min and products were isolated using a G50 Sephadex spin column (Roche Diagnostics, Indianapolis, IN). Biotin labeling was confirmed as described [16]. DNA was digested with BamHI, PstI, or ScaI to create single free 5' protruding, 3' protruding, or blunt ends, respectively, and then gel purified.

2.2 Protein purification

Ku protein was expressed using baculovirus vectors and purified as described [17]. SFPQ•NONO were prepared to near homogeneity from human (HeLa) cells as described [5]. Fractions from the final Mono S chromatographic step were used except where otherwise indicated. SFPQ and NONO are the major polypeptide components of the purified fraction (refer to Fig. 1 of ref [5]) and have been shown to be essential for its end-joining stimulatory activity. DNA-PKcs was prepared as described except that the phenyl-Superose and Mono S chromatography steps were omitted [18].

2.3 DNA binding assays

DNA binding assays were performed in Reacti-BindT streptavidin-coated polystyrene strip plates with Super BlockT (Pierce Biotechnology, Rockford, IL). All incubations were performed in a volume of 50 μl. Wells were incubated with 3% bovine serum albumin (Fisher Scientific, fraction V) in Buffer DB (100 mM KOAc, 20 mM Tris-HCl (pH 7.9), 0.5

mM EDTA, 10% glycerol, 10 µg/ml phenylmethylsulfonylfluoride, and 1 µg/ml each of soybean trypsin inhibitor, aprotinin, leupeptin, pepstatin A) for 2 h at room temperature. Wells were washed 3 times with Buffer DB buffer, and biotinylated DNA (0.12 pmol) was added and incubated for 1 h. In assays using doubly-blocked DNA, additional streptavidin (0.5 µg) was added and incubation was continued for 30 min. Test proteins were added and incubated for 1 h. After washing to remove unbound proteins, primary antibody, diluted in blocking buffer (1% bovine serum albumin, 0.02% γ-globulin in buffer DB) was added and incubated for 3 h at room temperature or overnight at 4 °C. Primary antibody was either monoclonal anti-Ku80 (mAb 111, Abcam, Cambridge, MA) or anti-SFPQ•NONO patient serum (kind gift of Dr. Yoshihiko Takeda, Georgia Regents University). Wells were washed three times with buffer DB containing 0.05% Tween 20. Alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG, or anti-human IgG, Sigma Chemicals, St. Louis, MO), diluted 1:30,000 in blocking buffer, was added and incubation was continued for 3 h at room temperature or overnight at 4 °C. Wells were washed three times in buffer DB with Tween 20 and twice in buffer DB without Tween 20. Wells were developed with a freshly prepared solution of 100 µl of KPL Bluephos substrate (KPL laboratories). Incubation was continued for 30 min and absorbance was measured at 595 nm. Assays were performed in duplicate and error bars indicate range.

2.4 DNA capture assay

Assays were performed using Reacti-BindT streptavidin-coated polystyrene strip plates. Radiolabeled DNA substrate was the same as described previously for end-joining reactions [5] and was prepared by digestion of pUC19 with BamHI, treatment with alkaline phosphatase, and 5'-end labeling with polynucleotide kinase and [γ-³²P] ATP. Microwells were loaded with DNA as described above. Separately, a mixture of proteins and radiolabeled capture DNA substrate was prepared in buffer DB. The mixture was transferred into the microwell and incubated 30 min at room temperature. Wells were washed three times with 0.5X buffer DB and retention of label was measured by liquid scintillation counting and reported as counts per minute (cpm).

2.5 DNA-PKcs activity assay

DNA-PKcs activity assays were performed essentially as described [19]. Phosphorylation reactions contained, in a volume of 10 µl, 25 mM Tris-HCl, pH 7.9, 25 mM MgCl₂, 1.5 mM DTT, 50 mM KCl, 10% glycerol, 20 nM pUC19 plasmid digested with BamHI, 0.16 µM [γ-³²P] ATP (6000 Ci/mmol), 10 nM DNA-PKcs, 20 nM Ku protein, and purified SFPQ•NONO complex as indicated in the figure legend. Reactions were incubated for 30 min at 30 °C. Reactions were terminated by addition of SDS-PAGE sample buffer. Products were analyzed by 10% SDS-PAGE and detected by PhosphorImager analysis (Amersham Pharmacia Biotech, Piscataway, NJ).

3. Results

3.1. Binding assay

We implemented a microwell-based protein-DNA binding assay. Linearized plasmid DNA was fill-in labeled with biotin-dUTP, cleaved, and gel purified to obtain singly biotinylated

fragments (Fig. 1A). These were allowed to bind streptavidin-coated microwells, then incubated with test protein. After washing, protein-DNA complexes were detected using primary and alkaline phosphatase-conjugated secondary antibodies (Fig. 1B).

We validated the assay by measuring the well-studied Ku-DNA binding interaction. Reactions were performed with varying amounts of Ku and a fixed amount of DNA (Fig. 1C). Ku was from a recombinant source and has previously been validated in functional end-binding assays [4]. Ku binding was concentration dependent and approached saturation at 1.5 pmol per reaction, corresponding to a Ku:DNA molar ratio of approximately 12:1. Ku loads at DNA ends and translocates inward, allowing multiple copies to bind to one DNA. Saturation presumably occurs when this cycle of binding and translocation reaches a limit.

We next tested SFPQ•NONO-DNA binding. SFPQ•NONO was native heterodimer purified from human cell extracts through five sequential chromatographic steps (refer to Materials and Methods). Binding was concentration dependent, similar to that of Ku (Fig. 1D). Saturation was not reached at the highest amount tested, which was 0.6 pmol per reaction, corresponding to a protein:DNA molar ratio of 5:1. This was the largest amount that could be tested, based on the stock concentrations of native protein.

3.2 Independent binding of Ku and SFPQ•NONO

We showed previously that Ku and SFPQ•NONO bind DNA together in an early step of the endjoining reaction and together form a committed pre-ligation complex [5]. It is not known if their binding is independent or cooperative. We found it difficult to distinguish these alternatives in a mobility shift assay, because of difficulty in assigning which proteins were present in heterogeneous mobility-shifted complexes. The microwell assay is based on immunodetection and is thus better suited for separate measurement of binding of different proteins in a mixture.

We performed binding reactions where Ku protein was varied and SFPQ•NONO was present at fixed concentration. There was very little difference in Ku binding, as reflected in absolute A_{595} levels, in the presence or absence of SFPQ•NONO (compare Fig. 1E with Fig. 1C). Moreover, SFPQ•NONO binding was only minimally affected by saturating levels of Ku (Fig. 1G). Analogous results were seen in reactions where the amount of SFPQ•NONO was varied and Ku was held constant (Fig. 1F, Fig. 1H). Together, data support a model where binding of Ku and SFPQ•NONO is largely independent.

3.3 Binding of SFPQ•NONO occurs in the absence of free DNA ends

Independent binding of Ku and SFPQ•NONO to DNA suggests that they probably occupy different sites. Ku is a sliding clamp that loads exclusively at DNA ends. Whether SFPQ•NONO binding similarly requires free ends is not known. To address this, we performed the microwell assay comparing a substrate where both ends were blocked by streptavidin-biotin complex with similar substrates where one end was free (Fig. 2A, 2B).

Ku was unable to bind to the substrate where both ends were blocked (Fig. 2C), as expected from prior solution studies using biotinylated oligonucleotides [20]. By contrast, Ku bound avidly to the substrates with one free end. Binding was similar whether the free end had a

5'-overhang, 3' overhang, or blunt structure, consistent with early observations (reviewed in [21]).

In contrast to Ku, SFPQ•NONO bound quite well to the substrate where both ends were blocked. Binding was comparable the substrates with a 5' overhang or blunt end (Fig. 2D). SFPQ•NONO bound somewhat more efficiently to the substrate with a 3' overhang than to the others, for reasons have not been investigated. Together, results demonstrate a clear difference between Ku and SFPQ•NONO: the former requires free ends for loading onto DNA, whereas the latter does not.

3.4 Ku and SFPQ•NONO have DNA capture activity

Binding of Ku to an immobilized DNA fragment promotes capture of a second DNA in solution [22, 23]. We investigated whether SFPQ•NONO had a similar activity, using the microwell-based assay format. A singly biotinylated DNA substrate was allowed to bind to streptavidin-coated microwells, then incubated with a mixture of test protein and a second, radiolabeled but non-biotinylated DNA fragment. After washing, the retention of the radiolabeled DNA in each well was measured.

We first validated the assay by measuring activity of Ku alone. As expected, Ku promoted capture of radiolabeled DNA in a concentration-dependent manner (Fig 3C). SFPQ•NONO also promoted capture of radiolabeled DNA, although ~4-fold higher concentrations were required for equivalent retention. We also tested the activity of Ku and SFPQ•NONO in combination. Increasing amounts of these two proteins were incubated in a fixed molar ratio of approximately 4:1, reflecting the relative activity when each protein complex was assayed individually. The activities of Ku and SFPQ•NONO were approximately additive under the conditions used.

3.5 SFPQ•NONO simulates DNA-dependent protein kinase activity

In the nonhomologous end-joining pathway of DNA repair, Ku recruits DNA-PKcs to form an active complex at DNA ends. DNA-PKcs then catalyzes trans-autophosphorylation, in a reaction that is thought to require synapsis of two DNA ends. If SFPQ•NONO promotes synapsis of DNA ends, it should increase DNA-PKcs autophosphorylation.

We tested the effect of crude SFPQ•NONO (Q-Sepharose fraction) or purified SFPQ•NONO (Mono S fraction) on DNA-PKcs autophosphorylation (refer to ref [5] for a more detailed description of the purity and end joining activity of MonoS fractions). We observed a several-fold stimulation. The crude fraction had somewhat more activity, suggesting that it either contains more SFPQ•NONO (the concentration of SFPQ•NONO in the crude fraction is not accurately known) or that it contains additional stimulatory factors. Results are consistent with a model where SFPQ•NONO stimulates DNA synapsis and thus DNA-PKcs autophosphorylation activity, although they do not exclude other mechanisms of kinase activation.

4. Discussion

We have developed and optimized a microwell-based protein-DNA binding assay and used it to further investigate the characteristics of SFPQ•NONO, a candidate DNA repair factor. Prior work suggested that SFPQ•NONO cooperate with Ku protein at an early stage to establish a committed pre-ligation complex [4]. We report four new findings: (1) SFPQ•NONO and Ku protein bind DNA independently, with little evidence of cooperativity or interference; (2) Ku requires free ends for binding but SFPQ•NONO does not; (3) DNA-bound SFPQ•NONO promotes capture of a second DNA fragment; (4) SFPQ•NONO stimulates DNA-PKcs autophosphorylation, consistent with formation of a synaptic complex.

Studies were performed using native preparations of SFPQ•NONO, which have previously been validated in end-joining assays. Immunoprecipitation and gel filtration experiments show that these form a highly stable heterodimer, with no evidence that free individual subunits are present in significant amounts *in vivo* [4]. Consistent with the biochemical data, a recent structure of a related protein complex reveals an extensive protein-protein interface involving the tandem RRM domains and an adjacent conserved region [24]. Although there have been prior reports of DNA pairing activity associated with the isolated SFPQ subunit [25–27], it is significant that such activity has now been associated with the same native complex that is active in functional end-joining.

Pairing or end-bridging activities have been separately reported for the Mre11•Rad50•Xrs complex, Ku, DNA-PKcs, and XLF [22, 23, 28–30]. In this context it seems likely that the end-joining stimulatory activity of SFPQ•NONO arises from an ability to cooperate with other repair factors to further stabilize a synaptic preligation complex. By binding to internal sites, SFPQ•NONO is able to carry out this function without occluding binding of other proteins, such as DNA-PKcs, to the termini proper. Consistent with this, SFPQ•NONO only minimally interferes with Ku binding, and stimulates (rather than inhibits) DNA-PKcs autophosphorylation.

It is noteworthy that the SFPQ•NONO complex lacks a conventional DNA binding motif. We had originally suggested that the tandem RRM motifs in SFPQ and NONO allowed binding to two DNA molecules simultaneously [5], in analogy to the distantly related horn A1 protein, for which a protein-DNA cocrystal structure has been determined [31]. However, it has since been reported that the isolated, proline glutamine-rich, low sequence complexity N-terminal region of SFPQ binds DNA *in vitro* [32] and that the same region contributes to relocalization of the SFPQ•NONO complex to sites of laser damage in cells [8]. Based on the sequence composition, this domain likely comprises an intrinsically disordered region (IDR). Such IDRs are commonly present in RNA binding proteins and transcription factors [33, 34]. We speculate that, in the native SFPQ•NONO complex, the N-terminal IDR contacts DNA, and a predicted C-terminal filament-forming motif [24] (present in both subunits) mediates inter-protomer interactions. Very recently, direct experimental evidence for the location of the DNA binding domain and for filament formation has been reported [35]. Further structure-function studies using recombinant proteins will be needed to test the contributions of these motifs to end-joining activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We investigated DNA binding of SFPQ•NONO, a nonhomologous end-joining factor
- Binding was independent of Ku protein, with little cooperativity or interference
- Unlike Ku, SFPQ•NONO does not require free DNA ends for binding
- DNA-bound SFPQ•NONO is able to capture a second DNA (pairing activity)
- SFPQ•NONO stimulates DNA-dependent protein kinase activity

Figure 1. Assay design and validation

A. Linearized, singly biotinylated plasmid substrate used in this experiment. **B.** Assay schematic. Biotinylated DNA was bound to streptavidin-coated microwells, proteins were added, and bound proteins were detected by ELISA. Preliminary experiments (not shown) were performed to identify optimal conditions for blocking the microwells, washing, and immunodetection of bound proteins. **C, D.** Binding assays using 0.12 pmol DNA and either Ku70•Ku80 or SFPQ•NONO (amounts in pmol). Detection antibody indicated. **E, F, G, H.** Binding assays using mixtures of Ku70•Ku80 and SFPQ•NONO (amounts in pmol). Detection antibody indicated. DNA was omitted from some reactions where indicated. Experiments in different panels were performed in parallel and are thus directly comparable.

Figure 2. Influence of DNA end structure on protein binding
A. Linearized plasmid substrates used in this experiment. **B.** Assay schematic, showing interaction between streptavidin-coated plate and singly or doubly-biotinylated substrates. **C, D.** Binding assays containing indicated proteins (amounts in pmol) and different DNAs (0.12 pmol of the following: blocked, both ends blocked; SE-5', single free end with 5' overhang; SE-3', single free end with 3' overhang; SE-blunt, single free end with blunt terminus. Detection antibody indicated.

Figure 3. DNA capture assay

A. Linearized plasmid substrates used in this experiment. Substrates were biotinylated or 5'- ^{32}P -labeled as indicated. Free 5' end of biotinylated substrate is cohesive with 5' ends of radiolabeled capture substrate. Binding assays contained SFPQ•NONO, Ku70•Ku80, or a mixture (amounts in pmol). Captured DNA was detected by liquid scintillation counting.

Figure 4. DNA-PK autophosphorylation assays. Assays were performed as described in Materials and Methods using indicated protein fractions. Molecular weight markers and positions of Ku70, Ku80, and DNA-PKcs are indicated. The concentration range for Mono S-purified SFPQ•NONO is 0–10 nM, comparable to that used in functional end-joining assays [5].