Supplementary Material for EMBO/2004/47425

Material and Methods

Molecular Biology

The human *PNK* cDNA was PCR-amplified from a human lymphocyte cDNA library and cloned into the pcDNA3.1/V5-His mammalian expression vector by TOPO-cloning (Invitrogen) yielding DDp436. The *PNK* open reading frame (ORF) and sequences encoding for the PNK FHA domain (amino acid residues 1-136) were amplified from DDp436 with flanking *Eco*RI restriction sites and cloned in-frame into the *Eco*RI site of pGEX-4T3 (Amersham) yielding DDp449 and DDp451. Arginine-to-alanine point mutations, corresponding to amino acid residue 35 in the *PNK* ORF, were introduced into the above plasmids to yield DDp437, DDp450 and DDp452. The human Xrcc4-pET15b plasmid was a generous gift from Dr. Stephen Jackson (Critchlow et al., 1997). From this plasmid, Xrcc4 was amplified and cloned into the pcDNA3.1/V5-His plasmid by TOPO-cloning to generate DDp438. The C-terminal Xrcc4 deletions corresponding to amino acid residues 1-179 ($Xrcc4^{179\Delta}$), 1-213 ($Xrcc4^{213\Delta}$) and 1-250 ($Xrcc4^{250\Delta}$) were generated from DDp438 (yielding plasmids DDp439-441), and $Xrcc4^{250\Delta}$ from Xrcc4-pET15b (yielding DDp455) by introducing a stop codon after the final amino acid. The threonine-to-alanine point mutations in Xrcc4 ($Xrcc4^{T233A}$, $Xrcc4^{T264A}$, $Xrcc4^{T282A}$, $Xrcc4^{T306A}$, $Xrcc4^{T321A}$, and $Xrcc4^{250\Delta T233A}$) were generated using the DDp438 or DDp439 plasmids as DNA templates yielding DDp442 to DDp446, and DDp457, respectively. The Xrcc4-pET15b and $Xrcc4^{250\Delta}$-pET15b plasmids were used as templates to generate $Xrcc4^{T233A}$ (DDp454) and $Xrcc4^{250\Delta T233A}$ (DDp456), respectively. All constructs were verified by sequencing. The human *Xrcc1* cDNA was PCR-amplified from human cDNA IMAGE clone ID: 6169231 (Open Biosystems) and cloned into the pcDNA3.1/V5-His mammalian expression

vector by TOPO-cloning (Invitrogen) yielding DDp447. Three threonine-to-alanine point mutations at positions corresponding to amino acid residues 453, 488 and 519 ($Xrcc1^{3XTA}$) were generated by Quikchange site-directed mutagenesis using DDp447 as a DNA template, yielding DDp448. Constructs were verified by DNA sequencing.

Expression and purification of fusion proteins

PNK and Xrcc4 recombinant proteins were produced in *E.coli* BL21(DE3)/pLysS (Novagen). For the purification of the GST-fusion proteins, bacteria were grown to an OD_{600} of 0.6 and expression was induced by addition of 0.2 mM IPTG for 3 hrs at 30^{o}C. Cell lysis and GST-fusion protein purification was done according to the manufacturer's protocol. Histidine-tagged Xrcc4 recombinant proteins were grown as above, but were induced with 1 mM IPTG at 37^{o}C for 2 hrs. Cells were lysed in a buffer containing 20 mM Tris-HCl pH 8.0, 0.5 M KCl, 20 mM imidazole pH 7.0, 5 mM β-mercaptoethanol, 10% glycerol, 0.2% Tween-20, and 1 mM PMSF and purified on nickel agarose beads (Qiagen). Proteins were eluted with 250 mM imidazole. The eluted fractions were then dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 1 mM DTT, and 10% glycerol and concentrated on a VS2 Vivaspin column (30,000 MWCO; Vivascience).

Transfections and survival assays

Transient transfections were performed with 2 μg of plasmid DNA using the Effectene transfection reagent (Qiagen). HEK293T cells were analysed for protein expression 36 hrs following transfection. To derive stable clonal transfectants, XR-1 cells were transfected with

pcDNA3.1/V5-His-derived plasmids and, 48 hrs post-transfection, cells were placed under selective pressure with 800 μg/ml of G418 (Gibco). Resistant colonies were isolated and maintained in F-12 media with 5% FBS and 200 μg/ml of G418. The expression of wild-type Xrcc4 or the Xrcc4 mutants in the clonal cell lines was confirmed by Western immunoblotting.

For clonogenic assays, cells were grown to confluency and were X-irradiated in a Faxitron X-ray cabinet (Faxitron) with 0-5 Gy, at 3 Gy/min, under aerobic conditions at room temperature. Treated cells were plated into 96-well plates at two different cell dilutions in triplicate. After 7 days, surviving colonies (colonies with greater than 50 cells were scored) were fixed and stained, then counted. Survival was expressed as a percentage of the relative plating efficiencies of irradiated to unirradiated control cells.

Immunofluorescence microscopy

For immunofluorescence experiments, cells grown on coverslips were fixed with cold (-20^{o}C) 100% methanol for 15 minutes, followed by cold (-20^{o}C) 100% acetone for 30 seconds on ice. All subsequent steps were performed at room temperature. The cells were blocked with 10% FBS in PBS for 30 minutes then incubated with anti-γ-H2AX monoclonal antibody (Upstate) or anti-V5 antibody (Invitrogen) for one hour, washed three times with PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 45 minutes. The cells were then washed twice with 0.1% Tween-20 in PBS, once with PBS, and DNA was stained with 4,6-diamidino-2-phenylindole. Fluorescence images were captured using a Nikon Eclipse E600 FN microscope, equipped with MetaMorph software.

Cell extraction and immunoblotting

For whole cell extracts (WCEs), cells were rinsed once in ice cold PBS, and lysed in 50mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 0.5mM DTT, 0.5mM EDTA, 1X Complete mini protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, and 50 mM sodium fluoride. For λ protein phosphatase treatment, WCEs were prepared as above but without addition of phosphatase inhibitors. All λ protein phosphatase incubations were performed at 30^{o}C for 30 minutes, and were stopped by the addition of 25 mM EDTA. For TBB treatment of HEK293T and XR-1 cells, 75 μM of TBB (Calbiochem) in DMSO was applied to cells 4 or 24 hours prior to lysis. Wortmannin (Sigma) treatment was accomplished by incubating HEK293T cells with 20 μM of wortmannin for one hour at 37^{o}C.

For immunoprecipitations, clarified WCEs were incubated with 1 μg of the relevant antibody on ice for 90 minutes with occasional gentle agitation. 20 μl of protein A or G-coupled sepharose beads were then added, and incubated for an additional hour at 4^{o}C. As indicated, WCEs were pre-treated with 50 μg/ml of ethidium bromide for 30 minutes on ice prior to immunoprecipitation. Immunoprecipitates were washed thrice with lysis buffer, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were transferred to PVDF membrane (Immobilon) and immunoblotted as indicated. Detection was performed using SuperSignal enhanced chemiluminescence (Pierce). For pull-down assays, 1.5 mg of WCE was mixed at 4^{o}C for 2 hours with glutathione sepharose beads bound with 1 μg of GST-fusion protein. The complexes were then washed, and analyzed for associated proteins by immunoblotting as described above.

Peptides and peptide binding studies

All peptides used in these studies were purchased from the Yale HHMI/Keck Biotechnology Resource Laboratory. They are (in single-letter amino acid code): Biotin-GGYDESTDEESEKK (T233), Biotin-GGYDESpTDEESE-KK (T233P, where pT denotes phosphothreonine); Fl-GGYNEEpTEVEHKK (7814, where Fl denotes fluorescein); Fl-GGYAAS-pT-DEESKK (7815) and Fl-GGYDESpTDEESKK (7816). For protein kinase assays, CK2 (Upstate) was used according to the manufacturer's instructions. For peptide pull-down assays, streptavidin magnetic beads (Dynal) were washed twice in binding buffer (PBS containing 0.2% NP40, 0.5 mM DTT, 500 μM AEBSF, sodium orthovanadate, and 30 mM β-glycerophosphate) and coupled to 20 ng of biotinylated peptides for 30 min at room temperature with gentle mixing in 500 μl of binding buffer. The coupled peptides were then washed twice with binding buffer, and incubated with 100 ng of purified recombinant protein in binding buffer (500 μl) with gentle mixing for 1 hr at 4^{o}C. The complexes were then washed thrice with binding buffer and resuspended in SDS-PAGE sample buffer. The bound proteins were detected by immunoblotting with a rabbit anti-GST polyclonal antibody (Z-5, Santa Cruz).

Isothermal titration calorimetry (ITC) was performed using a VP-ITC microcalorimeter (MicroCal) and analyzed using Origin software according to the manufacturer's instructions. A typical experiment involved serial 10 μl injections of phosphopeptide (400-500 μM) into a sample cell containing 20-40 μM of purified PNK^{FHA} protein in PBS. Measurements were performed at 25°C. For fluorescence polarization (FP) experiments, equilibrium binding constant determinations were carried out using on an Analyst HT System (Molecular Dynamics). The binding studies were conducted using equivalent fluorescent intensities of the labeled peptides while varying the concentration of the protein PNK^{FHA} from 0.001-50 μM. Reaction mixtures were allowed to equilibrate for 10 minutes at room temperature prior to each measurement. The

polarization assays were performed in PBS buffer, using a 96 well plate format with a total volume of 200 µl for each well. All FP measurements were carried out at 25^{o}C. Binding constants for the peptides were calculated using GraphPad Prism 3.0 Software.

SPOT array synthesis and analysis

Peptide arrays were generated according to the SPOTS-synthesis method. Standard FMOC chemistry was used. FMOC protected and activated amino acids were spotted onto acid-hardened cellulose membranes derivatized with polyethylene glycol using an AbiMed ASP422 robot. The amino acids were spotted at a concentration of 0.25 M and were twice spotted at a volume of 0.2 µl for each coupling reaction. Final side chain deprotection was achieved after 3 hours using a cleavage cocktail consisting of 82.5% trifluoroacetic acid, 5% thioanisole, 5% phenol, 5% water, and 2.5% 1,2 ethanedithiol (Sigma-Aldrich). All washing, FMOC and side chain deprotection steps were performed manually in polypropylene containers. The membranes were then blocked in 5% milk/TBST for 1 hour at room temperature, incubated with 10 µM PNK^{FHA} in 5% milk, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT overnight at 4^{o}C, and washed four times with TBST. The membranes were then incubated with rabbit anti-GST polyclonal antibody in 5% milk/TBST for one hour at room temperature, washed five times with TBST, and incubated with anti-rabbit HRP secondary antibody (Jackson ImmunoResearch) in 5% milk/TBST for 30 minutes at room temperature. Following five more washes with TBST, detection was performed using SuperSignal enhanced chemiluminescence (Pierce).

In vitro end-joining

Linearized plasmid DNA cut with *Bam*HI treated with or without calf intestinal phosphatase (New England Biolabs) was used as a substrate in all cohesive end ligation assays. Assays were performed with 200-400 ng of DNA substrate in 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 25 μg/ml BSA, 1 mM ATP, 10 mM $MgCl_2$, 1 unit of Xrcc4-DNA ligase IV complex (Trevigen) and increasing molar amounts of recombinant PNK^{WT} or PNK^{R35A} in a final volume of 20 μl for 30 minutes at 37°C. Reactions were stopped by the addition of 0.1% SDS and 10 mM EDTA, and deproteinated with proteinase K for 10 minutes at 37°C. Samples were separated by 0.8% agarose gel electrophoresis and stained with ethidium bromide. For the end-joining experiments employing peptides T233 and T233P, increasing molar amounts of the peptides were added to the same reaction conditions described above. PNK 5'DNA-kinase assays were performed using a similar set of conditions in the absence of the Xrcc4/DNA ligase IV complex, and with 4μCi/reaction of [γ^{32}P]-ATP added. The reaction products were deproteinated, resolved on 5% non-denaturing PAGE, and visualized using autoradiography.

Mass Spectrometry

In-gel digestion of proteins was performed on the Investigator ProGest Robot (Genomic Solutions) as described (Houthaeve et al., 1995). Samples were analyzed by an LC-MS system consisting of an Agilent HPLC System HP 1100, and an LCQ Deca Ion Trap Mass Spectrometer (Thermo Finnigan). Peptides were on-line washed and concentrated on C-18 custom-packed precolumn (Integrafrit CAPILLARY IS360-75-50-N-5) and separated by reversed phase HPLC on a PicoFrit Column PFC7515-PP18-5 (both from New Objective). Buffer A was 95 % Water, 5% acetonitrile, 0.1% Formic Acid. Buffer B was 90% acetonitrile, 10% Water, 0.1% Formic Acid. A flow rate of 250 nL/min was used to separate the peptides.

The column effluent was sprayed directly into the Mass Spectrometer. Spectra were searched against NCBI (Bethesda, MD) database with the help of Mascot (Matrix Science, London, UK).

Supplementary Figure Legends

Figure S1.

(A) Alignment of the C-terminal region of Xrcc4. Xrcc4 protein sequences from human (HsXrcc4, isoform 1; gi:12408649), mouse (MmXrcc4; gi:15054474) and rat (RnXrcc4; gi:34853018) were aligned by the ClustalX software of Vector NTI (InforMax). Five conserved threonine are boxed and their nomenclature follows that of their respective position on HsXrcc4 isoform 1.

(B) The PNK FHA domain is part of an FHA domain subfamily. Iterative PSI-BLAST searches with the PNK FHA domain identified among the most significant hits, the FHA domain of aprataxin (gi:28329436) and of an uncharacterized cDNA (MGC47799; gi: GI:27734905). These three FHA domains were introduced in an alignment of selected FHA domains (sequence details available upon request) and aligned using the AlignX program of Vector NTI (InforMax). The tree was generated without Kimura's correction and without considerering gaps.

Figure S2. Putative CK2 phosphorylation sites in Xrcc1 are critical in regulating the FHA-dependent PNK-Xrcc1 interaction.

(A) Whole cell extracts (WCEs) from HEK293T cells expressing V5-epitope tagged PNK, PNK^{R35A} or empty vector were immunoprecipitated with an anti-V5 monoclonal antibody. The immunoprecipitates or 20 μg of WCEs were separated on SDS-PAGE, and immunoblotted with

the anti-V5 antibody, to detect PNK proteins (bottom panel), or anti-Xrcc1 polyclonal antibody (top panel).

(B) HEK293T WCEs from cells expressing V5-epitope tagged wild-type Xrcc1, Xrcc1^{3XTA}, or empty vector were mixed in pull-down assays with recombinant GST-FHA (FHA PD, top panel). As a control, 20 μg of input WCE was loaded (bottom panel). The samples were resolved on SDS-PAGE and immunoblotted with a rabbit anti-Xrcc1 polyclonal antibody.

(C) HEK293T cells expressing the V5-epitope tagged constructs described in (B) were immunoprecipitated with anti-V5 monoclonal antibody. The immunoprecipitates or 20 μg of WCEs were resolved on SDS-PAGE and immunoblotted with a rabbit anti-PNK polyclonal antibody.

Figure S3. PNK stimulates the end joining of 5'-phosphorylated DNA ends.

Increasing amounts of purified recombinant PNK (0.1, 0.5 and 1 μg) was assessed in in vitro end-joining assays using a linearized and 5'-phosphorylated plasmid DNA substrate. Controls either lacking PNK, the Xrcc4/DNA ligase IV complex or both, are also show. The reaction products were separated on an agarose gel and stained with ethidium bromide.

Figure S4. DNA-PK is not required for the PNK-Xrcc4 interaction.

(A) HEK293T cells preincubated with or without 20 μM wortmannin for 1 hour at 37^{o}C were mock- or X-irradiated with 10 Gy. WCEs were then assessed in pull-down assays with PNK^{FHA}, and immunoblotted with anti-Xrcc4 antibody.

(B) WCEs from DNA-PKcs deficient cells (M059J) or the control cell line (M059K) were mixed with GST or GST-FHA in pull-down assays, and examined by anti-Xrcc4 immunoblotting. As a control, 20 μg of WCE input was also loaded.