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Impact of PNKP mutations associated with microcephaly, seizures and developmental delay on enzyme activity and DNA strand break repair

John J. Reynolds¹, Alexandra K. Walker¹, Edward C. Gilmore²,³, Christopher A. Walsh²,⁴ and Keith W. Caldecott¹,*

¹Genome Damage and Stability Centre, University of Sussex, Science Park Road, Falmer, Brighton, BN1 9RQ, UK, ²Division of Genetics, Manton Centre for Orphan Disease, Howard Hughes Medical Institute, Children’s Hospital, Harvard Medical School, Boston, MA 02115, ³Division of Child Neurology, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114 and ⁴Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA

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ABSTRACT

Microcephaly with early-onset, intractable seizures and developmental delay (MCSZ) is a hereditary disease caused by mutations in polynucleotide kinase/phosphatase (PNKP), a DNA strand break repair protein with DNA 5'-kinase and DNA 3'-phosphatase activity. To investigate the molecular basis of this disease, we examined the impact of MCSZ mutations on PNKP activity in vitro and in cells. Three of the four mutations currently associated with MCSZ greatly reduce or ablate DNA kinase activity of recombinant PNKP at 30°C (L176F, T424Gfs48X and exon15Δfs4X), but only one of these mutations reduces DNA phosphatase activity under the same conditions (L176F). The fourth mutation (E326K) has little impact on either DNA kinase or DNA phosphatase activity at 30°C, but is less stable than the wild-type enzyme at physiological temperature. Critically, all of the MCSZ mutations identified to date result in ~10-fold reduced cellular levels of PNKP protein, and reduced rates of chromosomal DNA strand break repair. Together, these data suggest that all four known MCSZ mutations reduce the cellular stability and level of PNKP protein, with three mutations likely ablating cellular DNA 5'-kinase activity and all of the mutations greatly reducing cellular DNA 3'-phosphatase activity.

INTRODUCTION

Discontinuities in one strand of the DNA double helix, denoted DNA single-strand breaks (SSBs), are the most commonly occurring type of DNA lesion in cells, with tens of thousands arising per cell per day (1,2). SSBs can arise either directly from the disintegration of damaged sugars or indirectly from abortive topoisomerase 1 activity or during the removal of damaged DNA bases or deoxyribose by DNA base excision repair. If not repaired quickly enough, SSBs can block transcription or result in the formation of DNA double-strand breaks (DSBs) during DNA replication (3,4). One source of SSBs is attack of DNA by reactive oxygen species (ROS) generated during normal cellular metabolism (5–7). To counter this threat, cells employ a number of DNA repair pathways collectively termed DNA single-strand break repair (SSBR) to ensure the rapid and efficient removal of SSBs (8).

SSBR typically involves four stages of repair: detection of the break, DNA end processing, DNA gap filling and DNA ligation. During DNA end processing, ‘damaged’ DNA termini are restored to conventional 3'-hydroxyl and 5'-phosphate moieties to enable subsequent steps of SSBR to occur. Enzymatically, this is the most diverse step because of the variety of types of damaged DNA termini that can arise. The importance of DNA end processing is indicated by the existence of neuropathological disorders in which proteins involved this process are mutated. These are ataxia oculomotor apraxia-1 (AOA1), spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) and microcephaly with early-onset intractable seizures and developmental delay (MCSZ), which are caused by mutations in apratxin (APTX), tyrosyl–DNA phosphodiesterase 1 (TDP1) and polynucleotide kinase/phosphatase (PNKP), respectively (9–12). A striking feature of these diseases is that they lack any major extraneurological symptoms, suggesting that the development and/or maintenance of neurones is hypersensitive to defects in DNA end
processing. There are notable differences between these disorders, however. For example, whereas MCSZ is primarily a developmental disease, associated with primary microcephaly and seizures from an early age, AOA1 and SCAN1 are progressive, degenerative, disorders.

MCSZ is the most recent disease identified in which the rate of SSBR is reduced. PNKP, the protein mutated in MCSZ, is a bi-functional enzyme that possesses both DNA 3'-phosphatase and DNA 5'-kinase activities. PNKP is a modular protein that possesses three distinct domains; an amino-terminal fork-head-associated domain (FHA) that mediates interactions with the SSBR and double-strand break repair (DSBR) scaffold proteins XRCC1 and XRCC4, respectively and the DNA phosphatase and DNA kinase catalytic domains (13–15). PNKP is a component of both SSBR and DSBR pathways and most likely operates at the majority of DNA breaks arising from attack of deoxyribose by ROS, because 50–70% of such breaks have 3'-phosphate termini (2.6). In addition, DNA strand breaks resulting from abortive topoisomerase I (Top1) activity or created by bi-functional DNA glycosylases during DNA base excision repair can also harbour 3'-phosphate termini (6,16,17).

To date, four mutations in PNKP have been identified in MCSZ (12). L176F and E326K are point mutations of highly conserved residues within the DNA phosphatase domain and T424Gfs48X and exon15 fs4X are frame-shift mutations in the DNA kinase domain that result in premature stop codons and truncated PNKP polypeptides (Figure 1). T424Gfs48X is the result of a 17-bp duplication within exon14 in the kinase domain, resulting in a 471 amino acid polypeptide of which the last 48 residues are out-of-frame. Exon15fs4X results in a 17-bp deletion within intron 15 that leads to mRNA transcript lacking exon 15, resulting in a polypeptide of 436 amino acids of which the last four residues are out-of-frame (12). E326K and T424Gfs48X are the commonest MCSZ mutations reported to date, with seven individuals homozygous for E326K and three homozgyous for T424Gfs48X. Additionally, four MCSZ individuals possessing the T424Gfs48X mutation are compound heterozygotes, harbouring the additional mutations L176F or exon15fs4X. Here we have analysed the impact of the MCSZ mutations on the specific activities of PNKP, to establish the molecular basis of this disease.

MATERIALS AND METHODS

DNA constructs

pET16b–PNKP encoding His-tagged human PNKP has been described previously (15). pET16b–PNKP L176F encodes a His-tagged phosphatase deficient PNKP mutant that was sub-cloned from pCD2-E/PNKD171N (18), pET16b–PNKP E326K, pET16b–PNKP T424Gfs48X and pET16b–PNKP exon15fs4X encode His-tagged PNKP proteins harbouring MCSZ mutations. pET16b–PNKP L176F and pET16b–PNKP E326K were created by site-directed mutagenesis of pET16b–PNKP using a QuikChange XL mutagenesis kit (Agilent Technologies) and the primer sets 5'-ATCTGGAGCGGA
Purification of recombinant proteins
His-tagged recombinant PNKP was expressed in the *Escherichia coli* strain BL21 (DE3) for 90 min at 20°C following the addition of 1 mM IPTG. Cells were sonicated in 50 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 10% glycerol, 1 mM DTT, 1 mM imidazole (pH 8.0), 1 mM PMSF and clarified by centrifugation. His-PNKP was purified by immobilized metal chelate chromatography (IMAC) using Ni–NTA Agarose resin (Qiagen), washing successively with wash buffer [50 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 10% glycerol, 1 mM DTT] containing either 20 mM (10 CV), 40 mM (10 CV) or 80 mM (10 CV) imidazole, and eluting in wash buffer containing 250 mM imidazole. Elution fractions containing His-PNKP were pooled, EDTA added to 10 mM to chelate eluted nickel ions (19), and dialysed in 50 mM Tris–HCl pH 7.5, 0.1 M NaCl, 10% glycerol, 1 mM DTT. With the exception of His-PNKPH176F, His-PNKP was then further purified by cation exchange chromatography and a 0.1–1 M NaCl gradient on a Mono S 5/50 GL column and AKTA Purifier UPC 10 system (GE Healthcare). Finally, elution fractions containing recombinant PNKP were dialysed [25 mM Tris–HCl, 150 mM NaCl, 10% glycerol, 1 mM DTT], aliquoted and snap frozen in liquid nitrogen and stored at −80°C.

Preparation of oligonucleotide duplex substrates
Oligonucleotide duplexes containing a SSB with a 1-bp gap and either 3’- and 5’-phosphate termini or 3’- and 5’-hydroxyl termini were prepared by annealing a 17-mer oligonucleotide (5’-TCCGTGAAAGCCTGCTT-3’) harbouring a 3’-phosphate or 3’-hydroxyl terminus (as appropriate) and a 25-mer oligonucleotide (5’-GACATACTAAGGTCGAACCGT-3’) harbouring a 5’-phosphate or 5’-hydroxyl terminus (as appropriate) with a 43-mer (5’-CCGTTTCGCATGCACTACGCTAGATCCATGCT-3’) harbouring a 5’-phosphate and 3’-hydroxyl terminus was also included to reduce degradation of 32P-labelled substrate by non-specific nucleases. Reactions were stopped and products analysed by phosphorimaging with the signal intensity of the labelled 25-mer (product) with the signal intensity of 32P-labelled 17-mer internal control.

Preparation of human lymphoblastoid cell-free extracts
Total cell extracts were prepared from 7.5 × 106 cells by lysis in 300 μl 20 mM Tris–HCl (pH7.5), 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton-X100 and 1/100 dilution of protease inhibitor cocktail (Sigma). Extracts were clarified by centrifugation at 14,000 rpm for 20 min at 4°C, aliquoted and snap frozen. Protein was quantified with a Bio-Rad DC Protein Assay Kit using BSA as a standard.

In vitro DNA phosphatase and DNA kinase reactions
For DNA phosphatase reactions employing recombinant PNKP, 25 nM of the indicated 32P-labelled substrate was incubated with the indicated concentrations of recombinant protein for 1 h at 30°C in 25 mM Tris–HCl pH 7.5, 130 mM KCl, 10 mM MgCl2 and 1 mM DTT. Reactions were stopped by addition of one-third volume of formamide gel-loading buffer and reaction products fractionated by 15% denaturing PAGE and analysed by phosphorimaging. PNKP phosphatase activity was quantified by measuring the signal intensity of the 17-P (substrate) and 17-OH (product) bands using Aida Image Analyser v.4.27 and calculating the percentage of substrate converted to product. For DNA kinase reactions employing recombinant PNKP, 25 nM of the indicated 32P-labelled substrate was incubated with the indicated concentrations of recombinant protein for 1 h at 30°C in 25 mM Tris–HCl pH 7.5, 130 mM KCl, 10 mM MgCl2, 50 mM ATP, 25 nM [γ-32P] ATP (6000 Ci/mmol) and 1 mM DTT. Reactions were stopped and products analysed as described above. PNKP kinase activity was quantified by phosphorimaging by normalizing the signal intensity of the labelled 25-mer (product) with the signal intensity of 32P-labelled 17-mer internal control. For reactions employing cell-free extract, reactions contained the indicated amounts of extract and 25 nM of the indicated 32P-labelled substrates and were incubated for the indicated time at 30°C in 25 mM Tris–HCl pH 7.5, 130 mM KCl, 1 mM DTT, 10 mM MgCl2. For reactions in which DNA gap filling or DNA ligation was measured, 100 μM dNTPs or 1 mM ATP, respectively, was included in the reaction buffer. A total of 1000-fold molar excess of a competitor oligonucleotide of unrelated sequence (5’-TC TGCAGCAGCTACCATC-3’) and harbouring a 5’-phosphate and 3’-hydroxyl terminus was also included to reduce degradation of 32P-labelled substrate by non-specific nucleases. Reactions were stopped and products analysed as described above.

For thermal inactivation DNA phosphatase assays, the indicated concentrations of recombinant PNKP were pre-incubated for 2 min 30s at the indicated temperatures and then incubated for 30 min with 60 nM of the appropriate 32P-labelled DNA phosphatase substrate as described above. For thermal inactivation DNA kinase assays, the indicated concentrations of PNKP were pre-incubated as above at the indicated temperatures and then incubated with 60 nM of the appropriate 32P-labelled DNA phosphatase substrate as described above.

Cell lines and cell culture
MC22601 and MC22602 are MCSZ lymphoblastoid cells (LCLs) and are compound heterozygous for the mutations T424Gfs48X and exon15Δfs4X. MC22604 and MC22606 are LCLs from unaffected relatives and are heterozygous carriers of the mutation exon15Δfs4X. MC5403 is a MCSZ LCL and is homozygous for the E326K mutation and MC5405 is a WT LCL from an unaffected relative. The 1635 is a LCL from an individual with SCAN1 and AG09387 is a WT LCL from an unrelated individual. MC22601, MC22606, MC22604, MC22606, MC5403 and MC5405 (12) and 1635 (20) have all been described previously, and AG09387 was kindly provided by Dr Mark O’Driscoll.

Fluorescence microscopy
A549 cells were seeded onto coverslips (1 × 104/cm2) and transfected 48 h later with pEYFP constructs encoding
WT or mutant PNKP using GeneJuice (Novagen) according to the manufacturer’s instructions. After 24 h, transfected cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% triton. Nuclear DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) and YFP positive cells were examined by fluorescent microscopy.

**Antibodies and immunoblotting**

Cells (2 × 10⁶) were lysed in hot (90°C) SDS–PAGE loading buffer, fractionated by SDS–PAGE, and transferred to nitrocellulose for immunoblotting with anti-PNK polyclonal antibody (SK3195) at a concentration of 1/1000 in 1% milk overnight at 4°C.

**Alkaline comet assay**

LCLs (2 × 10⁵ cells/sample) were mock treated (DMSO vehicle) or treated with 14μM CPT for the indicated time periods at 37°C, or alternatively were mock irradiated or γ-irradiated (20 Gy) on ice and incubated at 37°C for the indicated repair periods. Chromosomal DNA strand breaks were then quantified using alkaline comet assays and Comet Assay IV software (Perceptive Instruments) as previously described (21).

**RESULTS**

To date, four PNKP mutations have been identified in MCSZ (Figure 1) (12). To analyse the impact of these mutations on the DNA 3'-phosphatase and DNA 5'-kinase activities of PNKP, recombinant His-PNKP containing the MCSZ mutations was expressed in *E. coli* and purified by cation exchange chromatography and/or immobilized metal chelate affinity chromatography (Figure 2A and B; Supplementary Figure S1). We noted that whereas PNKP<sup>E326K</sup>, PNKP<sup>T424Gfs48X</sup> and PNKP<sup>exon15Afs4X</sup> exhibited similar chromatographic properties to WT PNKP during purification, PNKP<sup>ΔT17IN</sup> failed to bind to a cation exchange column (Supplementary Figure S1). As a negative control for DNA phosphatase activity, we also expressed and purified in parallel the protein PNKP<sup>ΔT17IN</sup>, which harbours a mutation in the DNA phosphatase catalytic domain and retains little or no DNA phosphatase activity (15,23).

To measure DNA 3'-phosphatase activity we employed a [32P]-radiolabelled oligonucleotide duplex that harboured a 3'-hydroxyl and 5'-hydroxyl termini containing 3'-hydroxyl and 5'-hydroxyl termini (Figure 3A, top). Phosphorylation of the 5'-terminus of this substrate by recombinant PNKP, in the presence of [γ-32P] ATP, was indicated by the appearance of a [32P]-labelled 25-mer product (Figure 3A and B, left). Note that the pre-labelled [32P]-17-mer in this substrate served as an internal loading control. As expected, and in agreement with previous studies, PNKP<sup>ΔT17IN</sup> possessed normal levels of DNA 5'-kinase activity (Figure 3A and B) (22–24). In contrast, PNKP<sup>T424Gfs48X</sup> and PNKP<sup>exon15Afs4X</sup>, which contained large deletions within the DNA kinase domain, possessed very little DNA 5'-kinase activity (Figure 3A). DNA 5'-kinase activity was also significantly reduced in PNKP<sup>ΔT17IF</sup>, despite this mutation being located in the DNA phosphatase domain (Figure 3B). However, PNKP<sup>E326K</sup> displayed near-normal levels of DNA 5'-kinase activity (Figure 3A).

Next, we examined the impact of MCSZ mutations on PNKP activity in whole-cell extracts prepared from MCSZ lymphoblastoid cells (LCL). For this study, LCLs from three MCSZ patients and three unaffected relatives were available for analysis (Figure 1B) [previously described in (12)]. MC22601 and MC22602 are LCLs from MCSZ siblings that are compound heterozygotes for the mutations T424Gfs48X and exon15Afs4X, and MC22604 and MC22606 are LCLs from unaffected relatives that are heterozygous for the mutation exon15Afs4X. MC5403 is from an individual with MCSZ that is homozygous for the E326K mutation, and MC5405 is from a WT unaffected relative. Cells from MCSZ individuals harbouring PNKP<sup>ΔT17IF</sup> are currently not available. In agreement with our previous report, all of the LCLs from individuals with MCSZ possessed greatly reduced levels of PNKP protein, retaining 5–10% of the normal level (Figure 4A) (12). Consistent with this, the three MCSZ cell extracts exhibited greatly reduced 3'-phosphatase activity on single-stranded and duplex DNA substrates at 30°C, as measured by the appearance of dephosphorylated (17-OH) product, compared to extracts from WT or heterozygous individuals (Figure 4B and C). Notably, however, all of the MCSZ extracts possessed some phosphatase activity, as suggested by the appearance of an 18-mer product in reactions containing duplex substrate and high concentrations of mutant cell extract, which reflected DNA polymerase activity on the nascent 3'-hydroxyl termini and filling of the 1-bp gaps (Figure 4C).

To measure DNA 5'-kinase activity in LCL extracts we employed an oligonucleotide duplex harbouring a nick with a 5'-hydroxyl terminus present on a 25-mer (Figure 4D, top). Phosphorylation of the 5'-hydroxyl terminus on the 25-mer was measured indirectly in these experiments, by the appearance of a 43-bp product resulting from ligation of the 32P-labelled 17-mer and the phosphorylated 25-mer. We failed to detect any DNA ligation product in reactions containing MCSZ cell extract from either MC22601 or MC22602 LCLs (Figure 4D), consistent with our observation that the mutant PNKP<sup>T424Gfs48X</sup> and PNKP<sup>exon15Afs4X</sup> proteins present in these cell extracts retain very little DNA kinase activity. In contrast, a small amount DNA ligation product was observed in reactions containing MCSZ cell
extract from MC5403 LCLs, suggesting that the greater residual level of PNKPE326K in these extracts was sufficient to support a low level of DNA kinase activity (Figure 4D).

Together, the experiments described above suggest that the mutant PNKP proteins present in MCSZ cells possess normal or near-normal levels of DNA phosphatase activity and also, in the case of PNKP E326K, DNA kinase activity, as measured in vitro at 30°C, but that these proteins are unstable in cells. While intracellular instability might be expected for the truncation mutants, it was more surprising for the point mutant PNKPE326K. To address this discrepancy, we examined the thermal stability of PNKP, PNKP<sub>L176F</sub> and PNKPE326K by pre-incubating the recombinant proteins at temperatures that spanned the physiological range (35–38°C) for a short period (2.5 min) prior to measuring their activity at 30°C. Consistent with the results above, PNKPE326K protein that was pre-incubated on ice exhibited near-normal DNA 3'-phosphatase (Figure 5A, left panels) and DNA 5'-phosphatase (Figure 5B, left panels) activity during
subsequent 30 min incubation at 30°C, whereas the activity of PNKP^L176F was partially reduced. However, both PNKP^E326K and PNKP^L176F were more sensitive to pre-incubation at 35°C and 38°C than WT PNKP, with PNKP^L176F exhibiting the greatest sensitivity (Figure 5A and B, middle and right panels). These data suggest that while the L176F and E326K mutations have relatively small effects on the specific activity of PNKP^L176F and PNKP^E326K at 30°C, they confer significant instability to PNKP and reduced activity at physiological temperature.

Finally, we compared the impact of MCSZ mutations on rates of chromosomal DNA strand break repair in living cells. To measure chromosomal DNA strand break repair rates we treated cells with \( \gamma \)-radiation, which induces both SSBs and DSBs of which \( \sim 70\% \) harbour 3'-phosphate termini, and measured total DNA strand breakage using alkaline comet assays. The MCSZ cell lines MC22601 and MC22602, harbouring PNKP^T424Gfs48X and PNKP^exon15Δfs4X, exhibited reduced DNA strand break repair rates following \( \gamma \)-radiation, as did the MCSZ cell line MC5403 harbouring PNKP^E326K (Figure 6A and B). Since none of the MCSZ mutations disrupt the nuclear localisation of PNKP [(25) and Supplementary Figure 2], these experiments suggest that the reduced total levels of PNKP protein and activity in MCSZ cells are sufficient to reduce the rate of chromosomal DNA strand break repair. We also examined the impact of MCSZ mutations on the repair of DNA breaks induced by camptothecin (CPT), a topoisomerase I (Top1) poison that results in DNA strand breaks (26) possessing both 3'-phosphate and 5'-hydroxyl termini (17,20).

Interestingly, while MC22601 and MC22602 MCSZ cell lines harbouring PNKP^T424Gfs48X and PNKP^exon15Δfs4X accumulated elevated levels of DNA strand breaks during CPT treatment, MC5403 MCSZ cells harbouring PNKP^E326K did not (Figure 5B and C). This suggests that
Figure 4. PNKP levels and activity in MCSZ lymphoblastoid cell extracts. (A) Western blot analysis of MCSZ LCL. Samples of the indicated LCLs (2 x 10^5 cells) were fractionated by SDS–PAGE, transferred to nitrocellulose and immunoblotted for PNKP. Quantification is shown on the right. The signal intensity of PNKP was quantified using Aida Image Analyser v.427 and normalized using the signal intensity of actin. Data are the mean ± SEM of six experiments. Note that, while not included here, PNKP protein levels in MC22601 were similar to its sibling LCL, MC22602. (B) Reduced DNA 3'-phosphatase activity in MCSZ LCL extract. 25 nM single-stranded substrate harbouring a 3'-phosphate terminus (top) was incubated with the indicated LCL extract (2.5 mg total protein) for the indicated time periods at 30°C. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. (C) Reduced DNA 3'-phosphatase activity in MCSZ LCL extract. 25 nM duplex substrate harbouring a 1-bp gap and 3'-phosphate terminus (top) was incubated with the indicated LCL extract (1, 2.5, or 7.5 mg total protein) for 60 min at 30°C. Reaction products were analysed as above. Note that DNA 3'-phosphatase activity is indicated by conversion of 17-P to 17-OH and DNA gap filling by conversion of 17-OH to 18-OH. (D) Reduced DNA 5'-kinase activity in MCSZ LCL extract. 25 nM duplex substrate harbouring a nick and 5'-hydroxyl terminus (top) was incubated with the indicated LCL extract (1, 2.5 or 7.5 mg total protein) for 60 min at 30°C. Reaction products were analysed as above. Note that DNA 5'-kinase activity is indicated indirectly in this experiment by appearance of 43-bp DNA ligation product.
different MCSZ mutations may impact on DNA strand break repair rates to a different extent, depending on the source and/or structure of the DNA break.

**DISCUSSION**

MCSZ is a human disorder associated with microcephaly, early-onset seizures and developmental delay (12). MCSZ results from mutations in PNKP, a DNA end-processing factor that possesses both DNA 3'-phosphatase and DNA 5'-kinase activities. To further understand the molecular defect behind MCSZ, the impact of the four disease-associated mutations identified to date on PNKP activity and rates of chromosomal DNA strand break repair was examined.

When analysed at 30°C, the truncated MCSZ proteins PNKP<sup>T424Gfs48X</sup> and PNKP<sup>exon15Afs4X</sup> each exhibited levels of DNA 3'-phosphatase activity comparable to
WT. In contrast, these proteins exhibited little DNA 5′-kinase activity, under the same conditions. The absence of significant DNA kinase activity in PNKPT424Gfs48X and PNKPexon15/C1fs4X is not surprising, because of their extensive deletions within the DNA kinase domain. However, it is surprising that the specific activity of the DNA phosphatase domain is normal, under the conditions examined, because in WT PNKP the phosphatase...
and kinase domains form a structural unit that is resistant to trypsin digestion (15). In the WT enzyme, the structural unit is stabilized by anchoring of the kinase domain to the phosphatase domain via interactions between the C-terminal five residues (Q517-G521 in human PNKP) and the interdomain linker/phosphatase domain (15). Intriguingly, while F518 is lost in PNKPT424Gfs48X and PNKPexon15Δfs4X, both mutants have acquired a new phenylalanine at their C-termini as a result of their frame-shift mutations (F469 in PNKPT424Gfs48X, which is 471 amino acids in length and F434 in PNKPexon15Δfs4X which is 436 amino acids in length). Perhaps these novel phenylalanine residues fulfil an analogous role to F518 in WT PNK, explaining their near-normal DNA phosphatase-specific activity at 30°C. Despite this, however, levels of PNKPT424Gfs48X and PNKPexon15Δfs4X protein and phosphatase activity are greatly reduced in MCSR cell extracts, most likely explaining the presence of disease pathology in affected individuals. Consequently, and since RT-PCR analysis failed to reveal nonsense mediated decay of PNKPT424Gfs48X or PNKPexon15Δfs4X mRNA (12), we suggest that while PNKPT424Gfs48X and PNKPexon15Δfs4X are stable at 30°C, they are unstable in cells and present at <10% of normal levels.

In contrast to the truncated mutant proteins, the point mutant PNKPE326K exhibited near-normal or normal levels of both DNA 3'-phosphatase and DNA 5'-kinase activity, at 30°C. Despite this, the clinical presentation of MCSR individuals harbouring the PNKPE326K mutation is no less severe than that of the other PNKP mutants (12). This discrepancy is likely explained by our finding that, similar to the truncation mutations, the E326K mutation results in 10- to 20-fold reduced cellular levels of PNKP protein. Since reduced PNKPE326K levels in MCSR do not appear to result from mRNA instability (12,27), it is likely that this point mutant results in significant cellular PNKP instability. Consistent with this idea, while PNKPE326K exhibited near-normal DNA kinase and DNA phosphatase activity at 30°C, the activity of this mutant protein was significantly reduced at 35–38°C. E326 forms hydrogen bonds with main chain amides of T323 and A334 and thereby helps correctly position the seventh α helix in the DNA phosphatase domain of PNKP (15,28). It is possible that lysine can be accommodated at this position at 30°C, but not at physiological temperature.

Of the four MCSR mutations identified to date, only PNKPL176F exhibited reduced levels of both DNA phosphatase and DNA kinase activity, at 30°C. L176 is part of an important hydrophobic pocket within the DNA phosphatase domain and is located near to the catalytic aspartate, D171 (15,28). The benzyl side chain of phenylalanine would most likely not be accommodated within this pocket and thus cause localized disruption of the DNA phosphatase domain, which in turn may lead to mis-folding of the DNA kinase domain. Consistent with this idea, PNKPL176F was the only mutant PNKP protein that displayed altered chromatographic properties during purification. This protein also exhibited significant temperature sensitivity, with both its DNA phosphatase and DNA kinase activity greatly reduced by incubation for short periods at physiological temperature. It thus seems likely that, similar to the other MCSR PNKP mutations, PNKPL176F levels will be reduced in MCSR cells harbouring this mutation. However, evidence for this awaits the availability of an appropriate MCSR cell line.

It is currently unclear whether it is reduced DNA kinase or DNA phosphatase activity, or both, that accounts for MCSR. All MCSR mutations examined to date result in >10-fold reductions in PNKP protein level, suggesting that both DNA 5'-kinase and DNA 3'-phosphatase activities are greatly compromised in all MCSR individuals. In addition, since we detected very little residual DNA kinase activity in recombinant PNKPT424Gfs48X or PNKPexon15Δfs4X proteins, even at 30°C, we suggest that individuals harbouring these mutants are effectively null for PNKP DNA 5'-kinase activity. Consistent with this idea, we failed to detect any DNA 5'-kinase activity in MCSR cell extracts harbouring these truncations. In contrast, all four of the MCSR mutations identified to date confer normal or near-normal levels of DNA phosphatase activity in the recombinant proteins at 30°C and, where examined, detectable levels of DNA 3'-phosphatase activity in protein extracts prepared from MCSR cells. Consequently, it is likely that PNKP DNA 3'-phosphatase activity is reduced >10-fold in all MCSR cells, it is unlikely to be null.

In agreement with their reduced cellular level of PNKP protein, all MCSR cells examined to date exhibit reduced rates of chromosomal DNA strand break repair following γ-irradiation, which induces DNA strand breaks of which ~70% possess 3'-phosphate termini. MCSR individuals harbouring the PNKPT424Gfs48X or PNKPexon15Δfs4X mutations also exhibited reduced rates of DNA strand break repair following treatment with camptothecin (CPT), a topoisomerase poison that induces SSBs harbouring both 3'-phosphate and 5'-hydroxyl termini. Interestingly, MCSR cells harbouring PNKPE326K exhibited normal DNA strand break repair rates following CPT. This may reflect the higher residual level of PNKP protein in MCSR cell extracts harbouring this mutation, compared to other PNKP mutations (Figure 5A). It is worth noting that the reduced rate of DNA strand break repair observed in MCSR cells following γ-irradiation most likely reflects reduced rates of SSB repair, because >95% of DNA breaks induced by γ-rays are SSBs (2,29). However, it is important to note that PNKP is also implicated in the repair of DSBs (30–33). Similar to SSBs, the majority of DNA termini present at DSBs induced by ROS are 3'-phosphate. MCSR may thus result from defects in both SSB and DSBR. Consistent with this idea, whereas microcephaly is a feature of the DSBR-defective disorders Lig4 syndrome and human immunodeficiency with microcephaly, seizures have been reported in patients with SCAN1 and in mice in which the SSBR protein XRCC1 is conditionally deleted in brain (11,34–37).

In summary, we report that all of the MCSR mutations examined to date result in 10-fold or greater reduced levels of PNKP protein, due most likely to reduced protein stability at physiological temperature. While two of the four mutations effectively ablate cellular PNKP DNA
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1 and 2.

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