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Structure–function defects of the twinkle amino-terminal region in progressive external ophthalmoplegia

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ABSTRACT

TWINKLE is a DNA helicase needed for mitochondrial DNA replication. In lower eukaryotes the protein also harbors a primase activity, which is lost from TWINKLE encoded by mammalian cells. Mutations in TWINKLE underlie autosomal dominant progressive external ophthalmoplegia (adPEO), a disorder associated with multiple deletions in the mtDNA. Four different adPEO-causing mutations (W315L, K319T, R334Q, and P335L) are located in the N-terminal domain of TWINKLE. The mutations cause a dramatic decrease in ATPase activity, which is partially overcome in the presence of single-stranded DNA. The mutated proteins have defects in DNA helicase activity and cannot support normal levels of DNA replication. To explain the phenotypes, we use a molecular model of TWINKLE based on sequence similarities with the phage T7 gene 4 protein. The four adPEO-causing mutations are located in a region required to bind single-stranded DNA. These mutations may therefore impair an essential element of the catalytic cycle in hexameric helicases, i.e. the interplay between single-stranded DNA binding and ATP hydrolysis.

1. Introduction

Mutations in nuclear-encoded proteins required for mitochondrial DNA (mtDNA) maintenance is an important cause of neurodegeneration and muscle diseases. The common result of these defects is either mtDNA depletion or accumulation of multiple deletions of mtDNA in postmitotic tissues. Mutations in many different genes have been shown to cause mtDNA instability, e.g. genes encoding for thymidine phosphorylase (ECGF1), thymidine kinase 2 (TK2), deoxyguanosine kinase (DGUOK), succinyl-CoA synthetase (SUCLA2), MPV17, p53-inducible ribonucleotide reductase (RRM2B), adenine nucleotide translocator 1 (ANT1), the mtDNA helicase TWINKLE, and the two subunits of mtDNA polymerase γ (POLγA and POLγB) [1–9]. Seven of the identified genes products, TP, TK2, DGUOK, SUCLA2, MPV17, and ANT1 probably influence the nucleotide pools and have indirect effects, whereas TWINKLE, POLγA, and POLγB are directly required for mtDNA replication.

The TWINKLE gene was originally identified in a search for mutations associated with chromosome 10q24-linked autosomal dominant progressive external ophthalmoplegia (adPEO), which is a human disorder characterized by exercise intolerance, muscle weakness, peripheral neuropathy, deafness, ataxia, cataracts, and hypogonadism [3]. Homology searches revealed a striking sequence similarity between TWINKLE and the bacteriophage T7 gene 4 protein (gp4) [3]. The gp4 protein contains both the DNA helicase and the primase activities needed at the bacteriophage DNA replication fork [10–12]. This observation immediately suggested that TWINKLE was the long sought replicative helicase in mammalian mitochondria. The TWINKLE helicase domain displays the same conserved organization as other members of the RecA/DnaB superfamily, and invariant amino acids of this superfamily are strictly conserved in TWINKLE [3,13,14]. Similar to the gp4 protein and related ring helicases, TWINKLE forms a hexamer in solution [3]. Recombinant TWINKLE also displays all of the classical features of a DNA helicase. The protein catalyzes the ATP-dependent unwinding of a DNA duplex with a distinct polarity (5′ to 3′), and it requires specific substrates with a single-stranded 5′-DNA loading site and a short 3′-tail to initiate unwinding [15]. The preferred substrate thus resembles the conformation of a DNA replication fork, a structure with which the TWINKLE protein would be expected to interact in vivo. The substrate requirement is also similar to what has been described previously for the gp4 protein and other hexameric helicases, such as DnaB [14]. The mitochondrial single-stranded DNA binding protein (mtSSB) has a stimulatory effect on the rate of DNA unwinding. In spite of this stimulatory activity, TWINKLE is unable to unwind longer stretches of double-stranded DNA (dsDNA) even in the presence of mtSSB. POLγ is unable to use dsDNA as template for DNA synthesis, but together POLγ and

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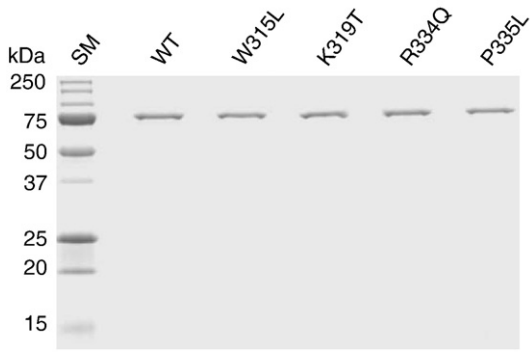


Fig. 1. Purification and characterization of the recombinant TWINKLE proteins. Purified recombinant wt and mutant versions of TWINKLE (0.5 μg) were separated by SDS-PAGE and revealed with Coomassie brilliant blue staining. lane 1, size marker; lane 2, wt; lane 3, W315L; lane 4, K319T; lane 5, R334Q; lane 6, P335L.

TWINKLE form a processive replication machinery, which can use dsDNA as template to synthesize long single-stranded DNA (ssDNA) molecules. DNA replication requires the presence of POLγB and addition of mtSSB stimulates the reaction even further, generating DNA products of about 16 kb, which corresponds to the size of the mammalian mtDNA molecule [16]. The presence of POLγA, POLγB, and TWINKLE in the minimal mtDNA replication machinery may explain why mutations in either of these components can cause adPEO.

The N-terminal domain (the primase-related domain) of human TWINKLE lacks several critical motifs required for primer synthesis in the T7 gp4 protein. A zinc-binding domain (ZBD) important for DNA binding is missing [17,18] and essential amino acids required for nucleotide polymerization are also absent [19,20]. In accordance with this observation, TWINKLE lacks primase activity in vitro (unpublished observations). Mitochondrial DNA replication is instead primed by the mitochondrial RNA polymerase [21,22]. Hence, in contrast to the primase domain of T7 gp4 protein that has been well-characterized biochemical, the exact function of the N-terminal part of TWINKLE (amino acids 43–348), is not understood in detail [23]. The N-terminal domain is required for efficient binding to single-stranded DNA and truncations of this region reduce both DNA helicase activity and mitochondrial DNA replisome processivity [24].

We and others have examined the molecular basis for adPEO mutations in the linker region and C-terminal domain of TWINKLE [25,26]. In the present study we have investigated the molecular phenotypes of adPEO-causing mutations in the N-terminal domain TWINKLE. We have expressed four adPEO-associated mutant versions of the TWINKLE protein with amino acid substitutions in the N-terminal domain [20]. The mutations display a dramatic decrease in ATPase activity, which can be partially overcome by the addition of single-stranded DNA. The mutated proteins have severe defects in DNA helicase activity and cannot support wild type levels of DNA replication in vitro. Our studies lead us to propose a model in which these adPEO causing mutations impair the interplay between single-stranded DNA binding and ATP hydrolysis, which is an essential element of the catalytic cycle of related hexameric helicases.

2. Materials and methods

2.1. Cloning

A C-terminal 6×His-tagged version of TWINKLE cloned into pBacPAK9 [16] was used for PCR based mutagenesis, as previously described [27]. Plasmids containing the W315L, K319T, R334Q, and P335L mutations were sequenced and used to prepare Autographa California nuclear polyhedrosis virus recombinant for the proteins as described in the BacPAK manual (Clontech).

2.2. Recombinant proteins

Recombinant POLγA, POLγB, mtSSB, and TWINKLE (wt and mutant versions) were expressed and purified as described previously [25,28].

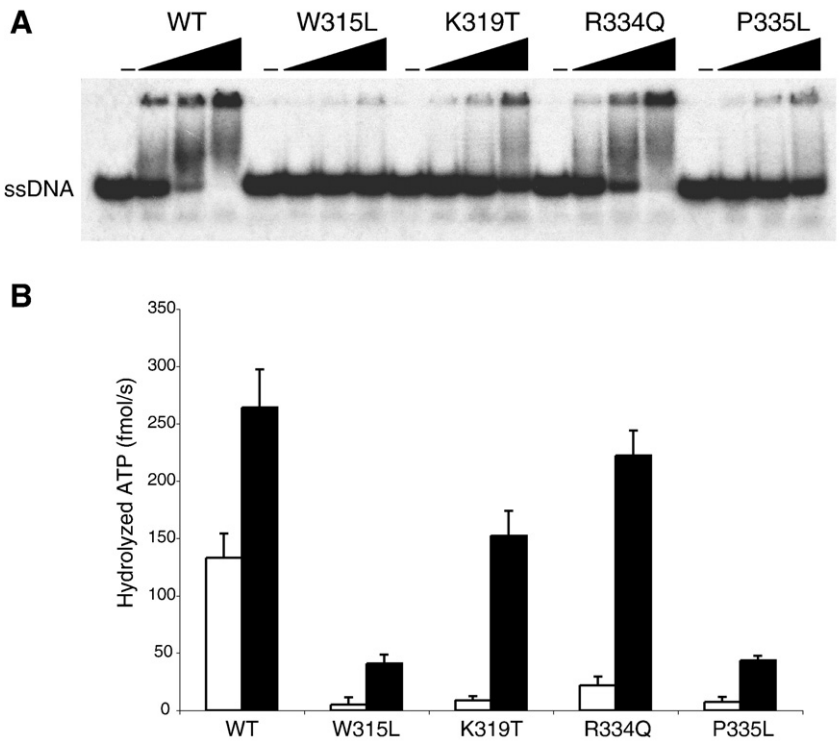


Fig. 2. AdPEO causing mutations have reduced ssDNA-binding and ATPase activity. (A) Gel-shift assays were performed as described in "Materials and methods" in the presence of increasing amounts of the different TWINKLE versions. (B) Wild-type TWINKLE and adPEO-causing mutants were analyzed for their ability to hydrolyze ATP in the absence (white bars) and in the presence (black bars) of M13mp18 ssDNA. An average of three independent experiments is presented.

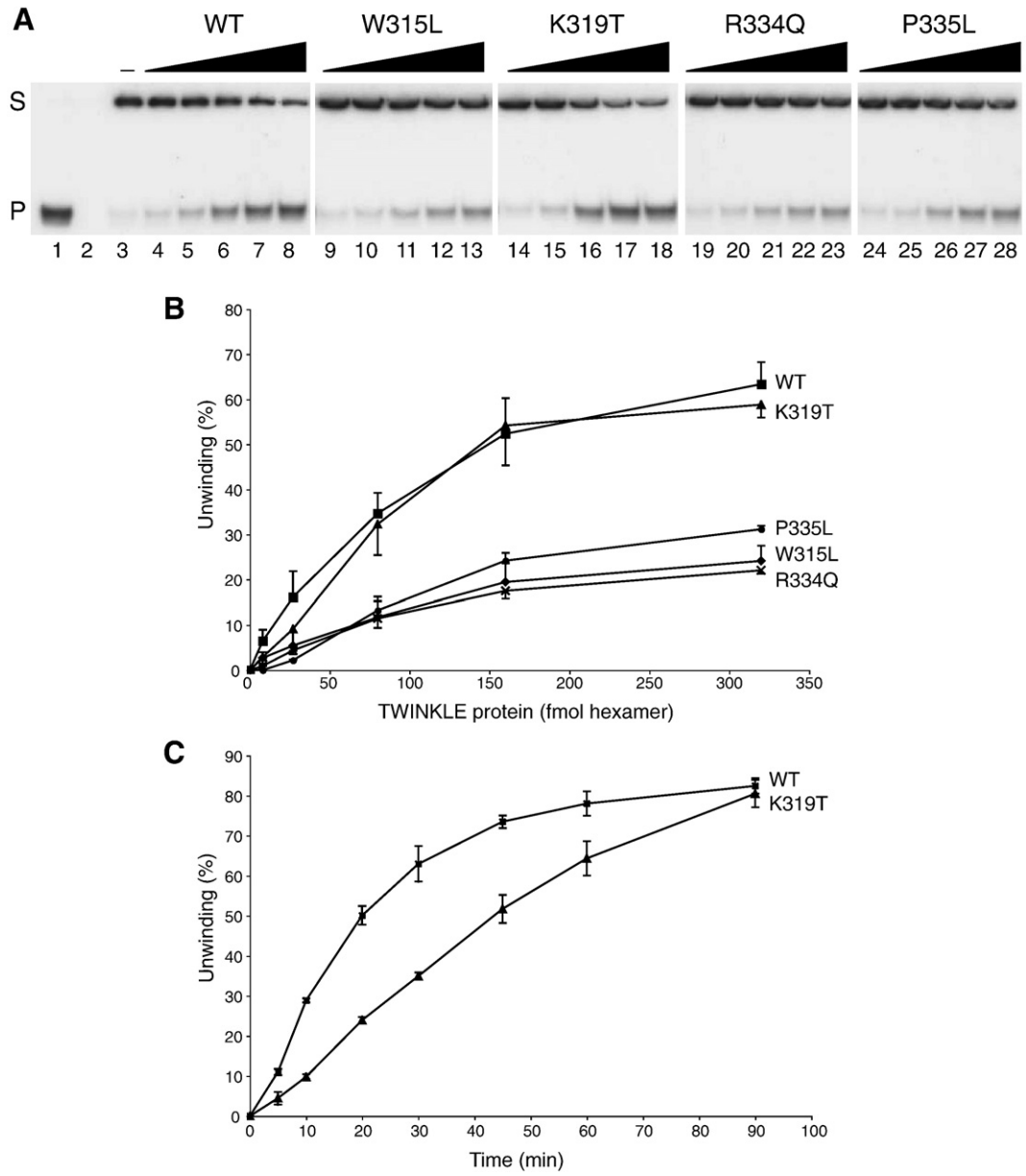


Fig. 3. AdPEO-causing mutations have reduced DNA helicase activity. Assays were performed as described in "Materials and methods". The DNA unwinding activity of wt and mutant TWINKLE was measured by monitoring the amount of unwound ssDNA product (P) from the duplex DNA substrate (S). (A) Increasing amount of TWINKLE protein was incubated with a constant amount of helicase substrate as described in Materials and methods. Lane 1, substrate heated to 100 °C before loading; lane 3, untreated substrate. (B) The helicase assay was performed exactly as in panel A and then analyzed with phosphorimaging and the progress of unwinding was estimated from the ratio of single stranded DNA product to the substrate. The data presented here is an average of three independent experiments. WT (■), W315L (♦), K319T (▲), R334Q (×), P335L (●). (C) A time course experiment was done in the present of constant amount of TWINKLE or K319T protein. At the times indicated, aliquots were removed and analysed described in Materials and methods. The gel was scanned and quantified as in panel B. WT (■) and K319T (▲).

The purity of the proteins was estimated by SDS-PAGE and Coomassie blue staining to be at least 95%.

2.3. Single-stranded DNA binding assay

Binding of TWINKLE to single-stranded DNA (ssDNA) was assayed using a 30-mer poly-dT oligo-nucleotide that had been radioactively labeled in 5′end with [γ-^{32}P] ATP using the T4 polynucleotide kinase (Stratagene). The reaction mixtures were 15 μl and contained 20 fmol DNA template, 20 mM Tris–HCl (pH 8.0), 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 10% glycerol, 2 mM ATP. Increasing amounts of TWINKLE (0, 50, 100 and 200 fmol, calculated as hexamer) were added as indicated in the figure legends. The samples were incubated for 10 min at RT before subjected to electrophoresis through a 6% polyacrylamide gel in 0.5× TBE for 15 min at 100 V.

2.4. ATPase assay

For ATP hydrolysis assays reactions mixtures were 20 μl and contained 20 mM Tris·HCl (pH 7.8), 10 mM NaCl, 1.5 mM $MgCl_2$, 17.4% glycerol, 0.3 mg/ml bovine serum albumin, 0.7 mM ATP, 300 nCi of [γ-^{32}P] ATP (GE Healthcare), and in the presence or absence of 188 fmol M13mp18 ssDNA. Wild-type or mutant TWINKLE protein (250 fmol) was added as indicated in the figure legends. Incubation was for 50 min at 32 °C. The reaction was stopped by addition of 400 μl of Norit A (12% in 0.1 M HCl, 10 mM K·PO_4) and analyzed as described earlier [15].

2.5. Helicase assay

DNA helicase assays were performed as described previously [15]. In Fig. 3A and B increasing amounts of the TWINKLE protein (8, 27, 80, 160, and 320 fmol, calculated as hexamer) was incubated with a constant amount of helicase substrate (5 fmol) for 40 min at 32 °C, before the samples were subjected to electrophoresis through a 10% native polyacrylamide gel. In Fig. 3C, a reaction mixture (120 μl) was prepared with constant amount of TWINKLE or K319T protein (2.7 pmol, calculated as hexamers) and template (30 fmol). The samples were incubated at 32 °C and at the times indicated, 15 μl aliquots were removed and analyzed by 10% non-denaturing polyacrylamide gel electrophoresis.

2.6. Rolling-circle DNA replication

To generate the mini-circle template for rolling-circle DNA replication in Fig. 4, a 70-mer oligonucleotide 5′-GGAATATTGAGGATGAAGGGTTGAGGTGAGTTGAGTGGAGTATAGGATCGGGAGGGTAGTATGGTGGAGG-3′ was circularized by using the CircLigase ssDNA ligase (Epicentre Biotechnologies). The circular product was annealed to a 90-mer oligonucleotide 5′-T_{40}CCTCCACCATAC TACCCTCCCGATCCTATACTCCACTCAACTCACCTCAA-3′. The mini-circle template (12.5 fmol) was added to a reaction mixture of 20 μl (25 mM Tris–Hcl (pH 7.5) 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 4 mM ATP, 10% glycerol, 250 μM dATP, 250 μM dTTP, 250 μM dGTP, 10 μM dCTP, 2 μCi [α-^{32}P] dCTP) in the presence of POLγ holoenzyme (100 fmol), mtSSB (5 pmol) and increasing amounts of the indicated versions of TWINKLE (0, 100, and 200 fmol, calculated as hexamers). The reactions in Fig. 4B were allowed to proceed for 2 h at 37 °C and analyzed on a denaturating 1% agarose gel as described earlier [16]. The experiment in Fig. 4C were performed as above but in the absence of mtSSB and the reactions were allowed to proceed for 1.5 h at 37 °C, before analysis on a 10% denaturating PAGE as described earlier [28].

2.7. Molecular modeling

Amino acid sequence alignment of TWINKLE (National Center for Biotechnology Information accession number NP_068602) with the T7 bacteriophage primase–helicase (National Center for Biotechnology Information accession number P03692) was carried out using the ClustalW multiple sequence alignment program as described earlier [25]. Homology modeling of TWINKLE was performed using the protein modeling server SWISS-MODEL in automated mode using the ClustalW alignment and chains A and B of the T7 primase–helicase heptamer and graphical visualization and analysis of the homology model were performed using Insight II and DS Viewer molecular modeling packages (Accelrys Inc., San Diego, CA) as described earlier [25].

3. Results

To investigate the molecular defects of adPEO-causing mutations in the N-terminal part of TWINKLE, we purified a series of mutant proteins in recombinant form, containing the following amino acid changes: W315L, K319T, R334Q and P335L. All four mutants were expressed (at about the same levels as the wt TWINKLE protein) and purified (Fig. 1). TWINKLE belongs to a family of hexameric helicases and analyses using minimally denaturing SDS-PAGE have demonstrated that the protein forms dimers, trimers, and higher order

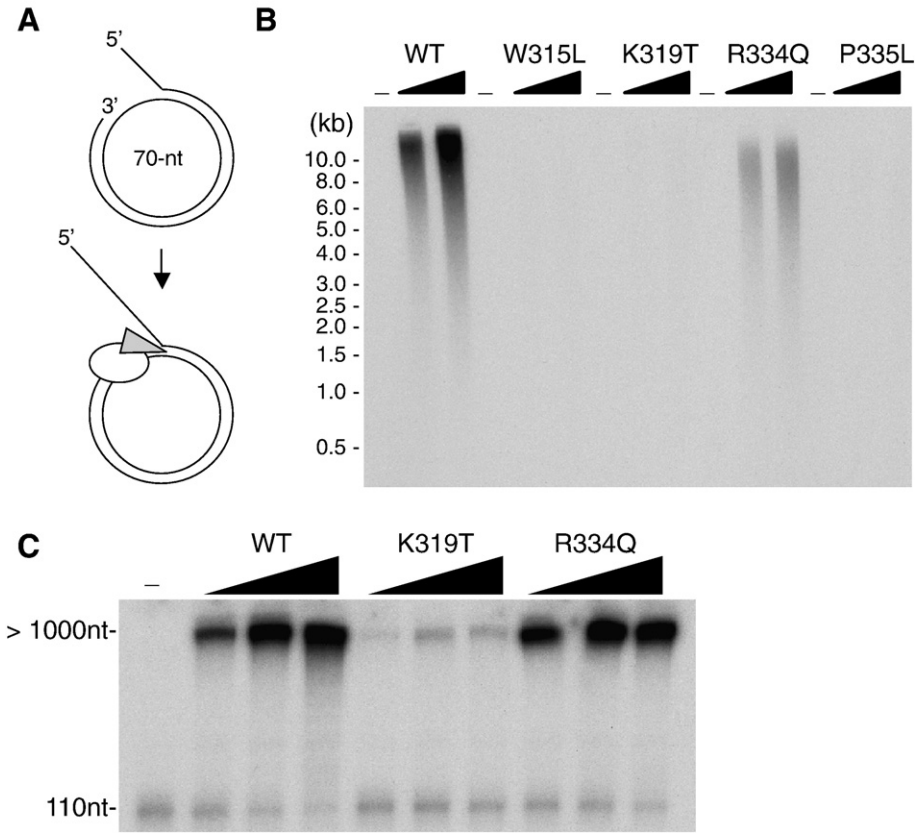
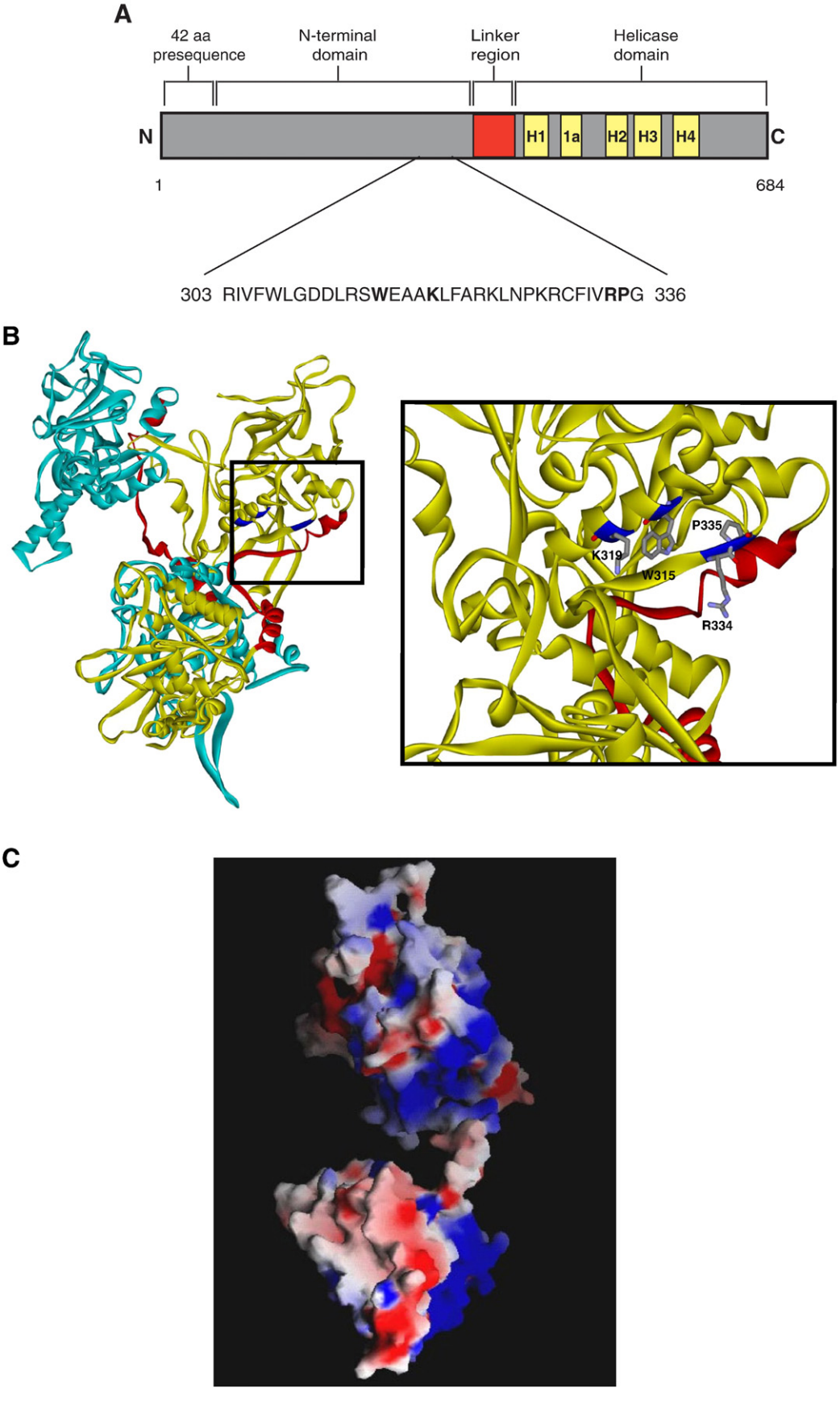


Fig. 4. R334Q support mtDNA replication in vitro. (A) The mini-circle template was generated as previously described [16]. The template can be efficiently replicated by the POLγ holoenzyme (white) and TWINKLE helicase (gray). The reaction is further stimulated by addition of mtSSB (not shown). (B) Rolling circle DNA replication assay was performed as described in Materials and methods in the presence of POLγ holoenzyme, mtSSB and increasing amount of indicated version of TWINKLE (C) Rolling circle DNA replication was performed as described in Materials and methods in the presence of only POLγ holoenzyme and increasing amount of indicated version of TWINKLE.


A
42 aa presequence
N-terminal domain
Linker region
Helicase domain
N
H1
1a
H2
H3
H4
C
1
684
303 RIVFWLGDDLRSWEAAKLFARKLNPKRCFIVRPG 336
B
P335
K319
W315
R334
C

multimers [3]. We recently demonstrated that amino acids substitution in the linker region and in the C-terminal part of TWINKLE that cause adPEO could affect the ability of TWINKLE to multimerize [25]. We wanted to investigate if the mutations in the N-terminal region of TWINKLE also could affect multimerization and therefore analyzed the mutant proteins with size exclusion chromatography as described earlier [25]. In contradiction to amino acids substitution in the linker region and in the C-terminal part of TWINKLE, our result demonstrated that the amino acid substitutions W315L, K319T, R334Q and P335L do not affect TWINKLE multimerization (data not shown). This result is in agreement with the finding that a truncated version of TWINKLE (Δ1–314) is still able to form hexamers [24].

3.1. Binding to single stranded DNA

TWINKLE only binds ssDNA in hexameric form and that the N-terminal domain contributes to the ssDNA-binding activity [24]. We now investigated if the mutations in the N-terminal domain may affect the ability of TWINKLE to bind ssDNA. A radioactively labeled 30-mer poly-dT oligonucleotide was used in order to avoid secondary structures that may influence the binding properties, and protein–DNA interactions were monitored in a gel-shift assay (Fig. 2A). The K319T, P335L, and W315L mutants all displayed severely reduced ssDNA-binding capacity. In contrast, the R334Q mutant bound to ssDNA with similar efficiency as wild-type TWINKLE.

3.2. ATPase activity of TWINKLE mutants

Unidirectional translocation of helicases on ssDNA is coupled to the hydrolysis of NTP (or dNTP) to NDP (or dNDP) and Pi [14]. Consequently, measurements of the ATPase activity of the different TWINKLE variants could give an indirect measurement of their ability to move on ssDNA and hence to unwind duplex DNA. The ATPase activities of the different TWINKLE mutants were measured both in the presence and absence of M13 mp18 ssDNA. ATP hydrolysis by wt TWINKLE is stimulated only about 2-fold in the presence of ssDNA (Fig. 2B). In the absence of ssDNA, all the four mutants in the N-terminal domain displayed reduced ATP hydrolysis activity, ranging from 4% to 16% of the intrinsic activities measured for the wt TWINKLE protein under the same conditions (Fig. 2B). However, in the presence of ssDNA, the ATPase activity of the mutants was stimulated 4 to 15 times. In fact, addition of ssDNA allowed the K319T and R334Q mutants to hydrolyze ATP with nearly the same activity as the wild-type protein (58% and 84%, respectively). The observed deficiency in ATP hydrolysis was unexpected, since the W315L, K319T, P335L, and R334Q mutations are all located in the N-terminal domain, whereas amino acids known to be responsible for ATP binding and hydrolysis are located in the helicase domains of TWINKLE. Furthermore, ssDNA seems to stimulate the ATP hydrolysis of all mutants in spite of our finding that the K319T, P335L, and W315L all displayed severely reduced ssDNA-binding capacity (Fig. 2A).

3.3. DNA helicase activity

The TWINKLE protein can unwind shorter stretches of dsDNA (<20 bp) provided the presence of a DNA fork structure [15]. We investigated the dsDNA unwinding activity of the W315L, K319T, P335L, and R334Q mutant proteins (Fig. 3A and B). In this initial experiment, all the mutants, except K319T displayed a severe reduction in DNA helicase activity. However, we analyzed K319T further and observed a slower dsDNA unwinding rate, than for the wt TWINKLE protein (Fig. 3C). The poor unwinding activity of R334Q was unexpected, since R334Q hydrolyzed ATP as efficiently as wild-type TWINKLE in presence of ssDNA.

3.4. Functional interactions with POLγ

The POLγ holoenzyme requires active TWINKLE for DNA synthesis on a duplex DNA template [16]. We examined the ability of wt TWINKLE and the mutant derivatives to support DNA synthesis on a mini-circle dsDNA template with a preformed replication fork (Fig. 4A). We incubated the template with constant amounts of POLγ holoenzyme and mtSSB together with increasing amounts of the individual TWINKLE mutants. In the absence of TWINKLE, the POLγ is unable to perform rolling-circle DNA synthesis [16]. Addition of wt TWINKLE or the R334Q mutant enabled POLγ to catalyze DNA synthesis (Fig. 4B). The W315L, K319T, and P335L all failed to support DNA synthesis (Fig. 4B). The inactivity of W315L, and P335L is not surprising, since these two proteins lack ATPase and ssDNA binding activity. The drastic phenotype associated with K319T was however unexpected, since this protein unwinds dsDNA as efficiently as wt TWINKLE although with slower initial unwinding activity rate. Earlier studies have demonstrated that mtSSB has a stimulatory effect on the rate of TWINKLE-dependent DNA unwinding [15,16]. This effect appears to be specific, since a heterologous single-stranded DNA binding protein (*E. coli* SSB) cannot substitute for mtSSB. The observed specificity may be due to a direct interaction between mtSSB and the TWINKLE protein. Physical interactions between replicative helicases and their endogenous single-stranded DNA-binding proteins have been demonstrated in many other systems, e.g. the herpes simplex virus type 1 helicase–primase complex is specifically stimulated by the viral SSB, ICP8 [29].

To investigate if the K319T deficiency was due to a defect in mtSSB-dependent stimulation of TWINKLE, we performed the rolling-circle assay also in the absence of mtSSB (Fig. 4C). We observed impaired DNA synthesis also in the absence of mtSSB, demonstrating that the deficiency of the K319T mutation is not simply a consequence of inefficient mtSSB-dependent stimulation (Fig. 4C). We could therefore conclude, that even a very mild change in TWINKLE activity, as that observed for K319T may disturb the coordination with POLγ holoenzyme and impair mtDNA replication.

3.5. Molecular modeling of TWINKLE

To better understand the molecular basis for the observed molecular phenotypes, we used a previously published TWINKLE model [25]. This TWINKLE structure is derived from homology modeling based on the only primase–helicase for which the crystal structure has been determined, gp4 from bacteriophage T7. The overall sequence similarity between gp4 and TWINKLE is around 46% (with only a 15% sequence identity), but the majority of the mismatches are unfortunately in the N-terminal domain, which is at main focus of the present study (Fig. 5A). It is therefore difficult to draw specific conclusions about molecular details in the TWINKLE, but some general observations can however help us to understand the observed phenotypes. Fig. 5B highlights the modeled location of the amino acid residues in the N-terminal domain for the different mutations (blue). From the model it is apparent that these residues do

Fig. 5. A molecular model of a TWINKLE dimer. (A) Schematic representation of adPEO mutations investigated in this study. (B) Homology model of the wild-type human TWINKLE (residues 43–684) developed from hexameric helicase structures. The figure shows a TWINKLE dimer, with the two monomers indicated in cyan and yellow. The linker region (red) separates the N-terminal domain (upper) from the helicase domain (lower). The residues for which mutations have been carried out in the N-terminal domain are shown in blue and the same residues are shown in atomic detail (right) in thicker stick representation with carbon atoms in gray, nitrogen atoms in blue and oxygen atoms in red. Hydrogen atoms are omitted for clarity. (C) Molecular electrostatic potential is shown mapped onto the surface of a TWINKLE monomer. The potential ranges from −5.0 kT to 5.0 kT, with electropositive surface in blue, electronegative surface in red and neutral surface rendered in white color.

not interact directly with the neighboring helicase subunits. In agreement with our findings that all four mutants are able to hexamerize (data not shown), the structure reveals that the affected amino acids are not involved in dimerization (and hence hexamerization) of the protein. The molecular electrostatic potential surface of one the monomers of TWINKLE (Fig. 5C) shows that the N-terminal domain contains an elongated region of significant electropositive potential, which in structurally related gp4 is the binding site for electronegative ssDNA [30]. The residues W315, K319, R334 and P335 are all located in this region and may therefore bind ssDNA during the process of DNA replication. Supporting this notion, all the mutants except the R334Q are more or less defective in ssDNA binding (Fig. 2A). Structurally, a W315L mutant is devoid of a hydrogen bond donating ability and this amino acid substitution may therefore result in loss of important hydrogen bonding interactions. The P335 is located in a turn in the predicted secondary structure (Fig. 5B) and the P335L mutant may affect the conformation of this unique secondary structure, thus resulting in partial loss of observed activities.

The R334Q mutant retains nearly wild type ssDNA binding activity, while the K319T mutant results in a relatively weaker ssDNA binding. Both these mutations result in a loss of electropositive nature of the side chains. Nevertheless, the N-terminal domain, where these two residues are located, has a strong electropositive potential owing to other surrounding residues (Fig. 5C), which may explain the relatively modest effects of these mutations on the general molecular recognition of ssDNA in this site. K319T results in a shortening of the side chain and loss of a specific interaction, which may explain the observed reduction in DNA binding activity. The ability to form hydrogen bonds is preserved in the R334Q mutant, which may explain why this mutant has retained the ability to bind ssDNA.

4. Discussion

The primase domain of the gp4 protein contains a zinc motif and a catalytic core [17]. The catalytic core contains several charged residues that are required for nucleotide binding and primer synthesis, and mutations in this region result in the loss of binding affinity for ATP [30]. Amino acids required for gp4 primase activity are not well conserved in the TWINKLE protein [20] and we have not observed primer synthesis activity or ATP binding to the N terminal domain of TWINKLE in vitro. The adPEO-causing mutations in the N-terminal region of TWINKE must therefore affect other essential functions of the protein.

Mutational studies of prokaryotic primases have also identified catalytically important basic residues that cluster around a shallow cleft located within the catalytic core. These residues form an elongated region with significant electropositive potential, which is required for binding to ssDNA [30]. The structural model of TWINKLE reveals that this positively charged surface is conserved and that the adPEO causing amino acid substitutions W315L, K319T, R334Q, and P335L are all located in this region (Fig. 5B and C). In agreement with this observation, three of the mutations, W315L, K319T, and P335L, display reduced ssDNA-binding capacity.

Most hexameric helicases like gp4 and E1HD are monomeric proteins that only assemble into hexamers in the presence of ssDNA, NTP or dNTP. In contrast, TWINKLE forms stable hexamers even in the absence of ssDNA or NTP [24]. Our findings suggest that the ssDNA-binding region of TWINKLE plays an important structural role for ATPase activity. One of the most noticeable phenotypes of W315L, K319T, R334Q, and P335L is a dramatic reduction in ATP hydrolysis in the absence of ssDNA. This reduction can be at least partially overcome by the addition of ssDNA, e.g. in the absence of ssDNA, the ATPase activity of R334Q is only 16% of wt TWINKLE, but in the presence of ssDNA, the ATPase activity reaches nearly the same levels as for the wt TWINKLE protein (84%). Our interpretation of these findings is the following. For gp4 and E1HD, binding to ssDNA induces structural changes leading to hexamerization and activation of ATP hydrolysis. TWINKLE is already "pre-activated", i.e. forms stable hexamers resembling the ssDNA-bound form of gp4 and E1HD, even in the absence of ssDNA. However, the precise structure of the ssDNA-binding cleft is still important for ATPase and ultimately helicase activity. Mutations that perturb the structural organization of the ssDNA-binding region may thereby negatively influence ATPase activity. Conversely, addition of ssDNA may counteract the negative structural impact of such mutations and stabilize the active conformation. The structural organization of TWINKLE and other hexameric helicases supports this idea. In the X-ray structure of the gp4 protein, ATP binds at the interface between two monomers. This interface is created by amino acids from the linker region and the N-terminal parts of the helicase domain. In turn, the linker region interacts directly with amino acids in the ssDNA-binding region. This intricate web of interactions between the ssDNA-binding and linker regions may directly influence the structure of the linker region and thereby influence ATP-binding and ATPase activity. In support of this notion, a large number of adPEO causing mutations have been identified in the linker region, including amino acid substitutions A359T, I367T, and S369P.

Recessive TWINKLE mutations (compound heterozygous TWINKLE mutations A318T and Y508C, or homozygous mutation T457I) have recently been shown to cause mtDNA depletion [31,32]. In contrast, dominant mutations in TWINKLE have so far not been linked to mtDNA depletion, but instead cause multiple mtDNA deletions in various tissues. Why the phenotypes of recessive and dominant TWINKLE mutations should differ, remains obscure. It is possible, that severe dominant mutations that cause mtDNA depletion may not be transmitted as they will not allow the individual to reach the reproductive age or alternatively may have effects on fertility. Because of this, only recessive mutations severely affecting mtDNA synthesis rate, may be propagated through the germ line. In contrast, milder dominant TWINKLE mutations, which have minor effects on mtDNA synthesis rate and primarily cause deletions via e.g. increased mtDNA mutation rate or stalling of the replication machinery, may be transmitted through the germ line. Consistent with this suggestion recent studies has demonstrated that there is a strong purifying selection in the female germ line to get rid of mtDNA aberrations that severely impair respiratory chain function [33,34].

In future studies, we will attempt to characterize TWINKLE mutations leading to mtDNA deletions or depletions, in order to understand the molecular basis for the interesting differences observed between dominant and recessive mutants.

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