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Mutation P732L in human DNA topoisomerase IIβ abolishes DNA cleavage in the presence of calcium and confers drug resistance

Chrysoula Leontiou, Jeremy H. Lakey, Robert Lightowlers, Rozalind M Turnbull and Caroline A Austin*.

Institute for Cell and Molecular Biosciences, The Medical School, University of Newcastle upon Tyne, NE2 4HH, UK. (CL,JHL,RL,RMT,CAA) current address: ETH Zürich, Institute for Molecular Biology and Biophysics, ETH-Hoenggerberg, CH-8093 Zürich, Switzerland (CL).

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Running title page

Running title: DNA topoisomerase IIβP732L

Corresponding author: Professor Caroline A Austin, Lab 3011, Institute for Cell and Molecular Bioscience, The Medical School, University of Newcastle, Newcastle upon Tyne, NE2 4HH, UK.
Tel: 0191 222 8864
Fax: 0191 222 7424
Email: caroline.austin@ncl.ac.uk

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Introduction 620 words
Discussion 1029 words

Abbreviations:
Topo, DNA topoisomerase;
mAMSA, methyl N-(4'-(9-acridinylamino)-3-methoxy-phenyl) methane sulphonamide;
AMCA, methyl N-(4'-(9-acridinylamino)-phenyl) carbamate hydrochloride;
mAMCA , methyl N-(4'-(9-acridinylamino)-2-methoxy-phenyl) carbamate hydrochloride;
MLC, minimum lethal concentration.

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ABSTRACT

The anti cancer drug mAMSA targets human DNA topoisomerase IIβ. Here we report the first selection with mAMSA of resistant human topoisomerase IIβ. Random mutagenesis of human DNA topoisomerase IIβ cDNA, followed by selection in yeast for resistance to mAMSA, identified βP732L. This mutant was 10 fold less sensitive to mAMSA and cross resistant to other chemotherapeutic agents such as etoposide, ellipticine, mAMCA, AMCA and doxorubicin. βP732L is functional but has reduced strand passage activities and altered DNA binding compared to the wild type protein. It has drastically altered cleavage properties compared to the wild type enzyme. It cleaved a 40bp DNA substrate in the presence of magnesium but at different positions to the wild type protein. More strikingly, βP732L was unable to cleave the 40bp DNA substrate, a 500bp linear substrate or a 4.3kb supercoiled substrate in the presence of calcium ions. This is the first report of a topoisomerase II mutation abolishing the ability of calcium to support DNA cleavage, and provides evidence for metal ion requirement for the phosphoryltransfer reaction of topoisomerase II.

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Topoisomerases regulate DNA topology, for example during replication and transcription, positive and negative supercoils are generated and topoisomerases are necessary to regulate the degree of supercoiling generated by these processes. Topoisomerase II activity is essential for segregation of chromosomes during cell division and for maintaining chromosome structure during mitosis. As indispensable enzymes for cell cycle progression, they are a good target for chemotherapeutic drugs used in cancer therapy. Type II DNA topoisomerases (topo II) catalyse topological changes in DNA by passing a double helix through another in a reaction coupled to ATP hydrolysis. The mechanism of action of topo II enzymes involves cleavage of double stranded DNA followed by passage of a second duplex, and religation of the cleaved DNA duplex (Wang, 2002).

Humans have two topo II isoforms, termed α and β. We have shown that both human topo II isoforms are poisoned by the anti-cancer drug mAMSA both in vitro and in vivo and that they had similar DNA cleavage site determinants in the presence of this cytotoxic drug (Marsh et al., 1996; Meczes et al., 1997). mAMSA increases the formation of DNA cleavage sites by inhibiting the religation reaction (Robinson, 1991). Further evidence that mAMSA can target both isoforms has come from studies with a pair of murine embryo fibroblast (MEF) cell lines with and without topo IIβ (Errington et al., 1999). In another study where topo IIβ was transfected back into cells lacking topo IIβ, restoration of sensitivity to mAMSA was seen confirming topo IIβ as a target of amsacrine (mAMSA) (Dereuddre et al., 1997).

All topo II poisons stabilize the normally transient cleavage intermediates or cleavable complexes, this DNA damage triggers apoptosis (Tewey et al., 1984; Pommier et al., 1991). Mutations in topo II genes which impair the ability of the drug to bind to the enzyme or decrease the DNA binding and cleavage properties of topo II enzymes can cause drug resistance (Pommier., 1993).

Several model systems have been used to select mAMSA drug resistance mutations in topo II to elucidate how this drug interacts with its target. Two mutations in the genes encoding bacteriophage T4 topo II have been reported to give rise to mAMSA resistant T4 (Fredenreich et al., 1998). The yeast model system utilises a strain bearing a temperature sensitive topo II, that can be rescued at the non permissive temperature by a plasmid borne functional topo II. This system has been used to select mAMSA resistant mutations in both yeast topo II and

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human topo IIα. Mutations at two positions in yeast topo II were identified, K478A (within the conserved PLRG**K** motif) and A642G (Wasserman and Wang, 1994). Use of this system also identified two mutations in human topo II α that conferred resistance to mAMSA, R486K and E571K. R486 is in the conserved PL**R**GK motif and the mutation R486K has also been found in human cell lines reistant to mAMSA(Patel et al., 2000). In addition, several human cell lines that are resistant to mAMSA have been shown to bear a point mutation R486K in human topoisomerase IIα (Kubo et al., 1996; Lee et al., 1992; Hinds et al., 1991; Bakic et al., 1986.)

Here we report the first use of the yeast model system to select for mAMSA resistance in human topo IIβ. Randomly mutagenised plasmids encoding human topo IIβ were transformed into a *S. cerevisiae* strain and grown in the presence of mAMSA. htopo IIβ bearing a P732L mutation was selected. *In vitro* characterisation of P732L revealed that this mutation had distinct cleavage properties compared to the wild type, to our knowledge, it is the first mutation in a type II topoisomerase that is able to cleave DNA in the presence of magnesium ions but not calcium ions.

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MATERIALS AND METHODS

Reagents:

Chemicals and drugs. mAMSA was obtained from The NCI, Bethesda. AMCA and mAMCA were supplied by Prof. B.C. Baguley, Auckland Cancer Society Research Center, University of Auckland, New Zealand. Etoposide, Mitoxantrone, Doxorubicin and Ellipticine were purchased from Sigma. DNA sequencing was carried out by Lark Technologies Inc (Essex, U.K.). Oligonucleotides were ordered from Invitrogen (Paisley, U.K.). Radiochemicals were purchased from Amersham (Bucks, U.K) and enzymes from New England Biolabs (Hertfordshire, U.K.) or Promega (Southampton, U.K). kDNA was purchased from TopoGen (Ohio, USA).

Yeast strains and expression plasmids

JN394*t2-4*: *α ISE2 urs3-52 top2-4 rad52::LEU2* A yeast strain carrying a temperature sensitive mutation in the yeast topoisomerase II gene that makes yeast viable at 25°C but non viable at 35°C (Nitiss, 1994). JEL1Δ*top1*: A protease deficient *S.cerevisiae* strain for the expression of topo II: *α leu2 trp1 ura3-52 prb1-1122pep4-3 Δhis3::PGAL4*.

YEphTOP2βKLM: Carries the first 5 codons of the TOP2 gene fused to codons 46-1621 of the human TOP2β cDNA, (βwt)*(*Meczes et al., 1997*)*.

Hydroxylamine mutagenesis and selection in the presence of mAMSA

Random mutagenesis and selection for drug resistance was as previously described (Leontiou et al., 2004), except that the selecting agent was mAMSA at 190 μM (74.7 μg/ml), twenty times the IC_{50} value in yeast (Meczes et al., 1997). The wild type htopo IIβ (βwt) cDNA was mutagenised by exposure to hydroxylamine. This library of randomly mutated plasmids were then transformed into the yeast strain JN394 top2-4 in which growth at the non-permissive

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temperature is dependent upon a functional plasmid - borne topo II. Drug - resistant transformants were selected by exposure to 190μM (75μg/ml) mAMSA in liquid culture for 96 hours. The surviving yeast were plated on drug free plates and grown for 5 - 7 days at 35°C, approximately 500 colonies grew and of these 36 were resistant when re-streaked onto plates containing mAMSA. Plasmids were rescued from these resistant yeast transformants. Yeast strain JN394 top2-4 was re-transformed with these plasmids and the transformants were grown on plates containing mAMSA to confirm if their drug resistance was plasmid borne. Following retransformation only three of the 36 grew on plates containing 10μg/ml mAMSA, so only 3 of the 36 had plasmid borne resistance. To confirm that a single point mutation in YEphTOP2βKLM was the cause of the drug resistance phenotype a fragment exchange was performed (Leontiou et al., 2004). Fragment exchange of the 1674bp region that contained the C to T change, resulting in the βP732L mutation, into an unmutagenised vector, was performed. The fragment exchanged construct was transformed into the JN394 top2-4 strain. The temperature sensitive mutation in the endogenous topo II gene in this strain permits growth of the yeast cells at 25°C but not 35°C.

Drug sensitivity was determined via the minimum lethal concentration (MLC), following short term exposure to mAMSA in liquid culture or by continuous drug exposure by plating the yeast on agar plates containing drugs at a range of concentrations and incubation at 35°C for 3-5 days (Leontiou et al., 2004).

Protein expression and purification: Recombinant htopo IIβ proteins were expressed in the yeast strain JEL1Δtop1bearing plasmid YEphTOP2βKLM or YEphTOP2βP732L, recombinant proteins were produced as described previously (Austin et al., 1995).

Strand passage assays: Decatenation and relaxation assays were carried out in relaxation buffer [50mM Tris-HCl (pH 7.5), 0.5mM EDTA, 1mM DTT, 100mM KCl, and 30μg/ml

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BSA] with 1μg of supercoiled pBR322 plasmid DNA or 400ng of kinetoplast DNA, 2mM ATP and 10mM $MgCl_2$, as described previously (Austin et al., 1995).

DNA cleavage assays: The cleavage properties of βwt and βP732L proteins were tested by incubating the protein with a ^{32} P end labelled 40bp linear DNA substrate. The cleavage religation equilibria of βwt and βP732L were tested in the presence of 10 mM $MgCl_2$ or 10 mM $CaCl_2$ to test the non drug stimulated cleavage properties of the enzymes, and in the presence of mAMSA (10μg/ml) to compare the drug stimulated cleavage of βwt and βP732L. To calculate the relative cleavage under these conditions the amount of end labeled cleaved product in several experiments was quantified using filmless autoradiographic analysis using a Fujifilm BAS-1500 machine. The amount of cleavage with wild type protein in the presence of mAMSA and magnesium was taken as 100% and the cleavage under different conditions was calculated relative to this (Marsh et al., 1996).

Cleavage was also analysed on a 564bp end labelled fragment, on 4.3Kb linearised pBR322 and on supercoiled plasmid DNA. In all of the cleavage experiments the protein was in excess over the DNA. For example, in the 40bp cleavage experiments there was a 10 fold molar excess of protein over DNA (3 and 0.3pmoles, respectively), whilst in the supercoiled cleavage their was only a 2 fold molar excess of protein over DNA (1 and 0.5pmoles, respectively).

Reversal of Cleavage assays: A 564bp fragment of pBR322 was amplified by PCR and reversibility experiments were carried out as described in (Leontiou et al., 2004).

Surface Plasmon Resonance: The DNA binding properties of the βwt and βP732L proteins were tested by surface plasmon resonance on a BIAcore 2000. The DNA substrates used were

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a 40 bp linear oligonucleotide (the same substrate used for the cleavage assays), a synthetic 4 way junction substrate and a piece of bent DNA from the *Drosophila* genome. The linear 40 bp substrate contains a single mAMSA cleavage site and allows the binding of one topo II dimer. The 4wj substrate contains the sequence of the 40 bp linear substrate along two adjacent arms, with the cleavage site SPR straddling the point of strand exchange. The bent DNA substrate is a 189 bp AT rich region from the *Drosophila* genome that forms a bend.

Topo II - DNA binding was carried out in 50 mM Tris–HCl pH 7.7, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, 1 mM ATP, 0.05% (v/v) Tween p20 and 1% (v/v) Triton X-100. Protein samples were injected using the BIAcore function 'kinject' at a flow rate of 70 µl/min. The protein was injected for 60 s and the dissociation phase was recorded for 180 s, at 25°C. After injection the chip was regenerated by a 30 s pulse of 0.5% (w/v) sodium dodecyl sulphate followed by a 60 s injection of 0.5 M NaCl. The concentration range of protein used was 5–60 nM. When protein becomes bound to DNA on the sensor chip, the refractive index of the medium at the chip surface is affected. This alteration is referred to as a change in RU, which represents an indirect measure of the amount of bound protein.

The association and dissociation data were modelled and analysed by using BIAevaluation software, version 3.0 (Pharmacia Biosensor AB, Uppsala, Sweden). The association and dissociation phases were analysed for several protein concentrations. Data was fitted by using the numerical integration method. The residual plots and the chi squared values were used to assess the appropriateness of the various models available for analysing the sensor data. Typically values of $\chi^2<10$ were accepted.

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Topo II DNA binding data were produced for various protein concentrations and were fitted simultaneously, assuming the 1:1 Langmuir model. The Langmuir model assumes that the analyte is both monovalent and homogeneous and the ligand is also homogeneous and that binding events are independent. The binding of topo II to DNA follows first order kinetics: A+B↔AB. The fitting parameters provide estimates of both association and dissociation rate constants (k_a and k_d) and from these values the equilibrium constants K_A and K_D were calculated ($K_D=k_d/k_a$ and $K_A=k_a/k_d$) [Leontiou et al., 2003 and references therein].

Protein structure analysis

Protein structures were manipulated and displayed using QUANTA, release 4.1.1. (Molecular Simulations Inc) on a Silicon Graphics Workstation. Human DNA topoisomerase IIβ was modelled on the yeast crystal structure using the program Modeler (Sali and Blundell, 1993), as described in West et al (2000). The structure was used to model the effect of the proline to leucine mutation at position 732. The model was subjected to an energy minimisation cycle using Charmm (50 steps steepest descents). A new structure was created in which the P to L mutation had been created by the edit command of Quanta. This file was subjected to the identical energy minimisation procedure as before. The two files (energy minimised wild type and energy minimised P732L were superimposed and structural differences investigated). Visual inspection revealed small localised changes and a full RMS analysis of the structural differences was not considered necessary.

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RESULTS

Selection and in vivo characterisation of mAMSA resistant yeast transformants

To identify mutations in DNA topo IIβ that confer resistance to mAMSA, a forced molecular evolution approach was used. Three plasmids conferred resistance to mAMSA. Two of the plasmids (mAMSA 1 and 13) that supported growth on 10 μg/ml bore the same point change a C to T at base 1394 resulting in a glycine to aspartic acid substitution of amino acid residue 465 (G465D). One transformant, mAMSA 7 was also able to grow on plates containing 200μg/ml. This most resistant transformant, mAMSA 7 contained the plasmid YEphTOP2βKLM bearing a transition mutation C→T at position 2195bp, resulting in a proline to leucine amino acid change at residue 732, YEphTOP2βP732L. Here we report the detailed characterization of βP732L.

βP732L is resistant to all anti-topoisomerase II drugs tested

βP732L was able to complement the endogenous yeast enzyme at 35°C confirming that this mutated protein is functional in vivo. The level of drug resistance to the selecting agent mAMSA was assessed in two ways. It was quantified using the minimum lethal concentration method (MLC) (Nitiss, 1994). JN394 top2-4 were transformed with plasmid encoding βwt or βP732L, and were grown in the presence of different concentrations of mAMSA for 6 and 24 (Figure 1A and B). Figure 1 shows the percentage relative survival versus drug concentration, the drug concentration that gave 100% survival was taken as the MLC. The MLC for βwt transformants on mAMSA was 1.5μg/ml after 6 hours and 2.4 μg/ml after 24 hours (Figure 1A and B). Yeast transformants expressing βP732L survived at higher mAMSA levels, and thus these transformants were exposed to a higher dose range. The MLC for βP732L transformant on mAMSA was 14.8μg/ml after 6 hours and 24.8 μg/ml after 24 hours (Figure

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1A and B). Thus βP732L conferred 9.9 fold resistance to mAMSA compared to the wild type. This difference was found to be statistically significant when a paired, two tailed t test was employed ($p<0.05$).

Cross resistance to other drugs was tested qualitatively by continuous exposure to drug (Leontiou et al., 2004). JN394 top2-4 expressing either βwt or βP732L was grown on YPDA plates containing different drugs including mAMSA, AMCA, mAMCA, etoposide, ellipticine or doxorubicin. βP732L was found to be resistant to all the drugs tested, ranging between ~2 and ~50 fold (Table 1).

βP732L shows decreased strand passage activities compared to the βwt protein

Wild type and mutated proteins were expressed in yeast and the enzymes were purified to >95% homogeneity (Figure 2A). Purified βP732L and βwt proteins were assayed for strand passage activity by both decatenation of kDNA and relaxation of supercoiled plasmid DNA. βP732L had significantly reduced relaxation activity (56.5%±8.2) compared to the wild type protein and reduced *in vitro* decatenation activity (74.4%±29.3) compared to the wild type protein (Figure 2B).

The $MgCl_2$ and ATP dependence of βP732L for decatenation was also measured. Decatenation was carried out in the presence of increasing concentrations of $MgCl_2$. The βwt enzyme showed maximal decatenation activity between 10-40 mM $MgCl_2$ whereas βP732L showed a narrower range between 10-30 mM $MgCl_2$ for maximal decatenation activity, thus βP732L is slightly more sensitive to increased concentrations of magnesium (Figure 2C). Analysis of the ATP dependence showed that βP732L did not have an altered ATP requirement for *in vitro* decatenation of kDNA (Figure 2D).

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βP732L has altered DNA binding properties compared to βwt.

βwt bound to all three substrates tested (linear, 4 way junction and bent DNA) with comparable affinity, the K_D values were in the range of 1-3nM (Leontiou et al., 2003). For the wild type protein the data best fit the simple 1:1 (Langmuir) model for all 3 substrates. βP732L bound to 4 way junction DNA with similar kinetics to βwt, with a K_D of ~1nM (Figure 2E). However, unlike the βwt, βP732L binding to the linear and bent DNA substrates did not fit a Langmuir binding model, so no K_D values could be determined for these substrates.

βP732L protein has altered DNA cleavage properties compared to βwt

Cleavage of a 40bp oligonucleotide

The ability of the βwt and βP732L proteins to cleave a 40 bp linear DNA substrate was tested (Figure 3A). This substrate contains one mAMSA site that is cleaved strongly in the presence of mAMSA and weakly in its absence, to produce two cleavage products of 21 and 15 nucleotides (Marsh et al., 1996).

The βwt protein weakly cleaved the DNA substrate in the presence of magnesium ions (7.8±2.4%) (Figure 3A, lane 2) and addition of mAMSA enhanced the levels of cleavage by 12.8 fold (100%) (Figure 3A, lane 3). Cleavage in the presence of calcium ions was increased by five fold (42±7.7 %) (Figure 3A, lane 4), compared to magnesium alone (7.8±2.4%) (Figure 3A, lane 2). Cleavage was highest in the presence of calcium and mAMSA (376.4±97.6%) (Figure 3A, lane 5), approximately 50 fold higher than with magnesium and

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no drug (Figure 3A, lane 2), 9 fold higher than calcium alone (Figure 3A, lane 4) and ~4 fold higher than magnesium plus mAMSA (Figure 3A, lane 3).

Cleavage of the 40bp substrate by βP732L was very different to cleavage of this substrate by βwt protein, in the presence of Mg^{2+} ions, it produced a ladder of approximately 15 cleavage products (Figure 3A lane 6), not the expected two products of 15 and 21 nucleotides. The amount of cleavage by βP732L with magnesium was greater than βwt under the same conditions (Figure 3A lanes 2 and 6). Nor was this cleavage by βP732L further stimulated by mAMSA (Figure 3A lane 7), unlike βwt whose magnesium cleavage was stimulated 13 fold by mAMSA. Strikingly, βP732L was not able to cleave DNA in the presence of calcium ions (Figure 3A, lane 8), even when mAMSA was added in the reaction (Figure 3A lane 9). Cleavage of this 40 bp oligonucleotide by βwt can also be stimulated by etoposide. However etoposide did not further stimulate cleavage by βP732L on this 40bp substrate, the cleavage products gave the same pattern and intensity with or without drug (Figure 3B), indicating that βP732L cleavage of the 40bp oligonucleotide substrate in the presence of magnesium is not further stimulated by mAMSA or etoposide.

Cleavage of a 564bp fragment of pBR322

To further investigate the cleavage characteristics of βP732L a 564bp fragment of pBR322 was isolated by PCR and end labelled. βP732L did not produce the same size cleavage products as the βwt protein. Cleavage of the 564bp substrate was carried out in the presence of magnesium and mAMSA (Figure 3C, lanes 2 and 6). Under these conditions the βP732L protein (Figure 3C, lane 6), produced different cleavage products compared to βwt with magnesium and mAMSA (Figure 3C, lane 2). Cleavage of the 564bp substrate by βP732L

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was also carried out in the presence of calcium ions and mAMSA; βP732L was unable to support cleavage in the presence of calcium ions (Figure 3C, lane 7), unlike βwt under these conditions (Figure 3C, lane 3).

Cleavage with manganese and nickel was analyzed for βwt and βP732L (figure 3C, lanes 4, 5 and 8,9). Manganese and nickel supported cleavage with both βwt and βP732L. The βP732L cleavage with Mn or Ni was more promiscuous than βwt, as seen with Mg, cleaving in more positions resulting in a different cleavage pattern. βwt cleavage with nickel was less intense than with manganese, this difference was not seen with βP732L.

βP732L forms more stable cleavable complexes in the absence or presence of mAMSA compared with βwt

The salt reversal of the complexes on the 564bp substrate was assessed to determine the *in vitro* stability of the complexes with either βwt or βP732L (Figure 4). The level of cleavage with βwt with only magnesium was too low to accurately determine the reversal times, but salt reversal of βwt with magnesium and mAMSA is shown in Figure 4 A, the half lives of complexes were ~5 minutes and all the complexes had reversed within 20 minutes. In contrast, βP732L complexes in the presence of magnesium either with or without mAMSA (Figure 4 B and C, respectively) were all still present after 20 minutes, showing their stability was greater than βwt and this stability was not drug dependent.

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Cleavage of linearised 4.3Kb fragment of pBR322

βP732L cleavage of linearised 4.3Kb was tested, with both mAMSA and etoposide the cleavage pattern resembled βwt in the presence of magnesium (Figure 5A), on this substrate no cleavage was seen in the absence of drug (data not shown).

Cleavage of supercoiled plasmid substrate by βwt and βP732L in the presence of Mg^{2+} or Ca^{2+} To test if βP732L was able to cleave longer substrates in the presence of calcium we tested the magnesium and calcium cleavage of supercoiled plasmid DNA both in the presence and absence of mAMSA (Figure 5B). βwt cleaved weakly with magnesium (Figure 5B lane 2) and well with calcium (Figure 5B, lane 3), and with either magnesium or calcium mAMSA increases the level of cleavage (Figure 5B lanes 4 and 5). βP732L showed more cleavage than βwt with magnesium alone (Figure 5B lanes 6 and 2, respectively). No cleavage was seen with βP732L and calcium (Figure 5B lanes 8 and 9), in agreement with the experiments on the 40bp and 564bp linear substrates.

Modelling of the central breakage domains of wild type topo IIβ and βP732L

Type II topoisomerases have 3 structural domains, an N-terminal ATPase, a central breakage – rejoining domain composed of the A' and B' sub fragments and a C-terminal domain (Austin et al., 1995). Residue 732 lies in the A' subfragment, which spans residues 722 to ~1200 in human topo IIβ. The A' subfragment can be further subdivided into three sections. The first section of A' spans residues β722-858 and is termed the "CAP-like" domain as it contains a fold similar to one found in the catabolite activator protein and histone H5 (Berger et al., 1996). This portion of the A' subfragment also contains the active site tyrosine (Y820) responsible for forming the phosphotyrosine linkage with DNA. P732 lies at the end of the A'

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α1 helix within the CAP-like fold. An alignment of the "*CAP-like"* domain from human topo IIβ, human topo IIα, yeast topo II and E.coli gyrase A (Figure 6a) shows that this region is conserved between species. P732 is a highly conserved residue, a proline is found at the position equivalent to 732 in type II topoisomerases from bacteria to humans.

The sequence similarity between the central breakage and rejoining domain of human topo IIβ and Yeast topo II enabled us to model the human topo IIβ sequence onto the yeast structure (West et al., 2000; Berger et al., 1996). Residues 420-1063 of htopo IIβ monomer were modelled onto the yeast crystal structure (Enzyme classification 5.99.1.3, Primary accession number P06786). The computer program Modeller was used which is conservative in that modelled structures are based very closely on the template structure (Sali and Blundell, 1993). The mutation P732L was then created in the htopo IIβ model to estimate the structural effects of the mutation, local conformation changes are predicted (Figure 6b and 6d). The leucine residue at 732 adopts an altered position to the proline 732 in the modelled structure and the peptide backbone is shifted in the vicinity of 732 in the model of the mutated protein (Figure 6d). Thus the proline to leucine amino acid change at position 732 in the model is not predicted to have any large effects on the overall structure. This is consistent with small < 2Å alterations in backbone positions and similar alterations in some side chains, e.g. Lysine 739. In the model some new H bonds are formed (e.g. Lysine 739 gamma nitrogen to the backbone at residue 731 due to shifting of the backbone in this region. However, these all rely on the small changes being accurate to better than 1Å which is beyond the resolution of the original X-ray structure. The predicted small changes are consistent with the fact that the selection procedure utilised will only select functional enzymes, amino acid changes that result in large structural changes that inactivate the enzyme would not be isolated.

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DISCUSSION

Mutation βP732L confers *in vivo* drug resistance of ~10 fold to mAMSA in an MLC assay and was cross resistant to all the topo II drugs tested, including AMCA, mAMCA, etoposide, ellipticine and doxorubicin (Figure 1 and Table 1). βP732L is functional *in vivo,* as our selection screen is dependent upon complementation in yeast. Although βP732L was functional in vivo, its in vitro strand passage activity is reduced compared to the wild type. Binding of βP732L to linear DNA substrates was altered, but binding to a four way junction substrate was similar to the wild type enzyme (Figure 2E).

Our in vitro experimental data with βP732L protein indicates it has altered cleavage properties. Most strikingly calcium could not support cleavage by βP732L, unlike βwt. Cleavage by βP732L of a 40bp or 564bp linear substrate both showed the same differences, greater cleavage with magnesium alone, no drug enhancement and no cleavage with calcium. On the 40bp and 564bp substrates, cleavage occurred at more sites when compared with the βwt enzyme (Figure 3a, b & c; Figure 4). However, on the 4.3Kb linearised pBR322 the cleavage pattern appeared similar to βwt (Figure 5a). This suggest that the altered DNA binding seen on 40bp and 189bp bent DNA substrates may be affecting cleavage site specificity on the shorter DNA substrates. Cleavage reactions using supercoiled plasmid substrate confirmed that calcium did not support cleavage by βP732L on the full length plasmid, but magnesium did. Using the 564bp substrate, the cleavage products generated by βP732L were shown to be more stable than those with βwt. This suggests an alteration of the cleavage – religation equilibrium of βP732L, favouring cleavage over religation, producing longer lived cleavable complexes compared to βwt. This could account for the increase in cleavage products with magnesium in the absence of drug.

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Why does this proline to leucine change generate a protein whose DNA cleavage reaction cannot be supported by calcium and has an altered cleavage – religation equilibria? To address this question the position of P732 within the protein was analysed by modelling this region onto the yeast structure. Modelling suggested the leucine change caused the local peptide backbone in βP732L to shift position compared to βwt. Topoisomerase is however a very dynamic protein and the removal of a highly conserved proline may alter the local mobility during catalysis. This cannot be accurately modelled using the currently available crystal structures of parts of the yeast protein.

A number of residues in this region have previously been demonstrated to be important for enzyme catalysis. Site directed mutations have been made in this region of the yeast topoisomerase II (Liu and Wang, 1998). Alanine substitutions at YR690, YD697, YK700, YR704, or YR781 abolished *in vivo* function in a complementation assay. The equivalent residues in htopo IIβ are βR729, βD736, βK739, βR743 and βR820, four of which are very close to P732.

In the yeast protein mutating R690 to alanine completely abolished *in vitro* cleavage activity (Liu and Wang, 1998). Yeast arginine 690 is equivalent to R32 in Gyrase A, Noble and Maxwell suggest this arginine is actively involved in the phosphoryl transfer mechanism that enables topo II to cleave the DNA (Noble and Maxwell, 2002). The equivalent residue in htopo IIβ is R729, which is only 3 residues away from βR732, so local conformational changes in the P732L protein may affect the proposed role of this arginine in DNA cleavage.

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Phosphoryltransfer reactions have been proposed to involve two metal ions in their mechanism, the proposed metal binding sites have magnesium ions co-ordinated by acidic amino acid residues such as aspartic acid (D) and glutamic acid (E) (West et al., 2000). We proposed two potential metal ion binding sites in htopo IIβ co-ordinated to the aspartic acids found in the IMTDQD(Q/H)DGSH loop, D557, D559 and D561 (West et al., 2000). A similar acidic triad is also found in topoisomerase I and mutation of these residues reduces the number of magnesium ions bound per protein molecule (Lima, 1994; Zhu and Tse-Dinh, 2000). Noble and Maxwell have published a model for the phosphoryl transfer in DNA cleavage by DNA gyrase (Noble and Maxwell, 2002). In figure 6c we have adapted the Noble and Maxwell schematic diagram to represent human topo IIβ. βR729 is shown in light green in Figure 6c, and illustrates the proximity of residue 732 to the hypothesised location of the DNA, active site tyrosine and the two metal binding sites hypothesized to catalyse the DNA cleavage reaction. The predicted local conformational changes may account for the altered DNA binding to linear DNA observed by SPR. It also suggests an explanation for the cleavage in the presence of magnesium but not calcium ions. If the local conformational change alters the liganding geometry for the bound metal ions, such that calcium can no longer support the phosphoryl transfer reaction and the magnesium supported phosphoryl transfer reaction has an altered equilibrium favouring cleavage over religation. This could account for the cleavage complexes being more stable with magnesium and absent with calcium.

A simple explanation for the altered cleavage properties with magnesium such as the extensive cleavage with magnesium alone and the lack of drug stimulation of cleavage in the presence of magnesium on short DNA substrates and lack of reversibility would be the presence of a contaminating nuclease. This possibility is very difficult to exclude since the

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amounts of such a protein could be so low that polyacrylamide gel elctrophoresis or MALDI approaches would not be guaranteed to pick it up. However, we do not see this non drug stimulated cleavage activity with magnesium in β wt or in 4 other mutated proteins expressed using the same system and purified by the same protocol. In addition we do not see any DNA products that appear to be due to nuclease activity in any of the agarose gels used for activity assays.

A study in HL-60 cells indicated that an intracellular calcium buffer could reduce the DNA cleavage by topoisomerase II in the presence of either etoposide or mAMSA. This reduction correlated with decreased cytotoxicity (Aoyama et al., 1998). The absence of cleavage with calcium may account for the drug resistance seen in yeast with P732L. However, whether removal of calcium stimulated cleavage is an important mechanism of drug resistance in human tumors remains to be determined.

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Footnotes

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Correspondence address: Professor Caroline A Austin, Institute for Cell and Molecular Biosciences, The Medical School, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK. Email caroline.austin@ncl.ac.uk

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Figure 1: Effects of mAMSA on the viability of yeast strain JN394 top2-4 transformed with plasmids encoding recombinant βwt or βP732L at 35°C. Viable counts were determined after growth of yeast transformants in the absence or presence of mAMSA at the concentrations indicated for 0, 6 or 24 hours. The values are expressed as a percentage of viable counts measured for time 0, percentage of relative survival. The results are the average of three independent experiments. Error bars represent the standard error from the mean of three experiments. A shows the 6 hour exposure to mAMSA and B shows the 24 hour exposure, plots of JN394 top2-4 expressing βwt (▲) and JN394 top2-4expressing βP732L (■) are shown.

Figure **2A:** A 7.5% SDS-PAGE gel loaded with 1.5μg of βwt or βP732L, and stained with coomassie blue. **B:** Strand passage activity, histograms of the relative relaxation activity (□) and relative decatenation (■) for β wt and β P732L. βwt is set to 100%. Means were calculated from at least 3 experiments, error bars represent 1 S.D. from the mean. **C:** Effect of Mg^{2+} concentration on decatenation by βwt (■) and βP732L (▲). Means and standard deviations are derived from at least 3 experiments. **D:** Effect of ATP concentration by βwt (■) and βP732L (▲). Means and standard deviations are derived from at least 3 experiments. **E:** DNA binding of human topo II βwt and βP732L by surface plasmon resonance to 4 way junction DNA. The experiment was carried out at 25°C, the association phase was 80s. resonance units are plotted versus time.

Figure **3 A:** Cleavage of the ^{32} P-labelled 40-bp DNA substrate by βwt and βP732L. Cleavage was carried out in the presence of no protein (lane 1) or1.2μg of βwt (lanes 2-5) or βP732L (lanes 6-9), plus Mg^{2+} (lanes 2&6), Mg^{2+} and mAMSA (lanes 3&7) Ca^{2+} (lanes 4&8) and Ca^{2+} and mAMSA (lanes 5&9). Cleavage products were quantified by filmless

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autoradiographic analysis using a fujifilm BAS-1500 machine. This figure shows an individual experiment that is typical of at least 3 others.

Figure **3B:** Cleavage of the ^{32} P-labelled 40-bp DNA substrate by βwt and βP732L. Cleavage was carried out in the presence of no protein (lane 1) or1.2μg of βwt (lanes 2&3) or βP732L (lanes 4&5), plus Mg^{2+} (lanes 2&4), Mg^{2+} and etoposide (lanes 3&5). Cleavage products were quantified by filmless autoradiographic analysis using a fujifilm BAS-1500 machine.

Figure **3C:** Cleavage of the ^{32} P-labelled 564-bp DNA substrate by βwt and βP732L. Protein (1.2μg) was incubated with the 564bp ^{32}P-labelled pBR322 substrate for 30 min at 37°C in the presence of various cations and mAMSA, and either βwt (lanes 2-5) or β732L (lanes 6-9). Lane 1 no protein, lanes 2 & 6 Mg^{2+} + mAMSA, lanes 3 & 7 Ca^{2+} + mAMSA, lanes 4 & 8 Mn^{2+} +mAMSA , lanes 5 & 9 Ni^{2+} +mAMSA.

Figure **4**: An autoradiograph of a 564bp ^{32}P-labelled pBR322 DNA substrate. Following incubation with or without mAMSA, in the presence of 10nM $MgCl_2$, and the indicated protein. Reactions were terminated at the noted time points (0-20 min).

Figure **5A:** Cleavage of the ^{32} P-labelled 4.3-kb pBR322 probe in the presence of mAMSA or etoposide. Lane 1 no protein, lanes 2 &4 βwt, lanes 3 &5 βP732L, lanes 2& 3 with mAMSA and lanes 4&5 with etopooside.

Figure **5B**: Cleavage of supercoiled plasmid DNA by βwt and βP732L. Protein (4μg) was incubated with 1.5 μg of supercoiled plasmid DNA in relaxation buffer with either 10mM $MgCl_2$ or 10 mM $CaCl_2$ +/- mAMSA with either βwt (lanes 2-5) or βP732L (lanes 6-9) for 30min at 37°C. Control supercoiled plasmid (lane 1). Lanes 2 & 6 Mg^{2+} , lanes 3 & 7 Mg^{2+} + mAMSA, lanes 4 &8 Ca^{2+} , lanes 5 & 10 Ca^{2+} + mAMSA,

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Figure **6(A)** To shows an alignment of the "CAP" like region of human topo IIβ (β) compared to that from human topo IIα (α), yeast topo II (Y) and DNA gyrase A (A).

The proline equivalent to P732 is shown in light blue, the arginine equivalent to R729 is shown in green and the active site tyrosine is shown in yellow. **(B)** Shows a diagram of one monomer of human topo IIβ showing the location of the P732L mutation in pale blue, the active site tyrosine in yellow, and arginine 729 in light green. The location of acidic residues involved in metal ion co-ordination are also shaded. The **E**GDSA motif containing E477 is shown in dark green and the IMT**D**Q**D**(Q/H)**D**GSH loop, containing D557, D559 and D561 is shown in red. **(C)** Shows a schematic adapted from a model previously proposed by Noble and Maxwell (22). This shows a two metal ion mechanism for the phosphoryl transfer reaction involved in DNA cleavage by DNA topo II. The colouring is equivalent to that seen in Figure 5A and B. Figure 6**D** A stereo image of the region immediately surrounding residue 732, the wild type sequence is shown in blue. The mutant chain in green with the leucine side chain shown in red, above which the wild type proline ring can be seen. The residue numbering is that of human topoisomerase II beta -1 which is a 1621 residue protein, not human topoisomerase II beta -2 which is a 1626 residue protein (Q02880).

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Table 1
The drug sensitivity profile of JN394 top2-4 expressing βwt or βP732L in the presence of different cytotoxics at 35°C.

The yeast strain JN394 top 2-4 was transformed with recombinant plasmid encoding either βwt or βP732L. The transformed strains were tested for drug sensitivity to different concentrations of topo II poisons. Equal amounts of each transformant as determined by optical density were plated in a series of dilutions onto adenine supplemented yeast extract/peptone/dextrose with drug and were grown at 35°C for 5 days. After 5 days the extent of growth was assessed by comparing the number of yeast colonies to the wt. the results shown here are the amount of growth of yeast colonies: +++++, excellent growth (eg., good growth at all dilutions); ++++ very good growth; +++, good growth; ++, reasonable growth; +, poor growth; and -, no growth. Comparisons of the level of growth from several dilutions on plates containing several different drug concentrations allowed approximate fold resistance values to be determined. The experiment was performed in duplicate.

Table 1

Drug	Plasmid	1μg/ml	5μg/ml	10μg/ml	25μg/ml	50μg/ml	100μg/ml	200μg/ml	Resistance
mAMSA	YEphtop2βKLM	+++++	+++	+/-	-	-	ND	ND	~10 fold
	YEphtop2βP732L	+++++	+++++	+++	+++	+++	ND	ND	
mAMCA	YEphtop2βKLM	++++	-	-	-	-	ND	ND	~50 fold
	YEphtop2βP732L	+++++	+++++	++++	++++	++++	ND	ND	
AMCA	YEphtop2βKLM	++++	+/-	+/-	-	-	ND	ND	~10 fold
	YEphtop2βP732L	+++++	+++++	+++++	+++++	+++++	ND	ND	
etoposide	YEphtop2βKLM	ND	ND	++++	++++	++++	+++	++	~2 fold
	YEphtop2βP732L	ND	ND	+++++	+++++	++++	++++	++++	
doxorubicin	YEphtop2βKLM	ND	++++	++++	+/-	-	ND	ND	~4 fold
	YEphtop2βP732L	ND	+++++	+++++	+++++	+++++	ND	ND	
ellipticine	YEphtop2βKLM	ND	++++	+++	+	-	-	ND	~8 fold
	YEphtop2βP732L	ND	+++++	+++++	+++++	+++++	+++++	ND	

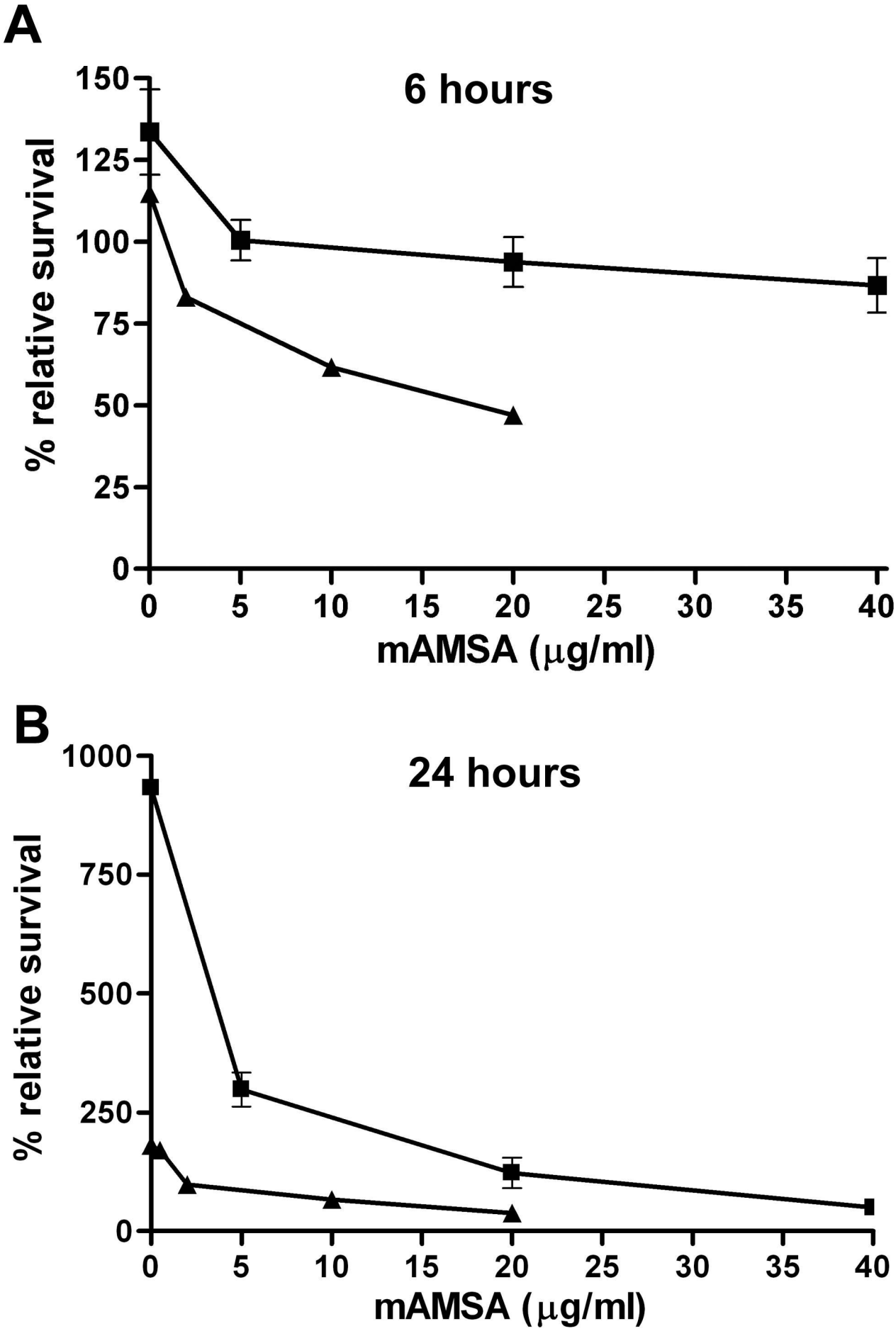

A
6 hours
% relative survival
150
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40
mAMSA (μg/ml)
B
24 hours
% relative survival
1000
750
500
250
0
0
5
10
15
20
25
30
35
40
mAMSA (μg/ml)

Figure 1

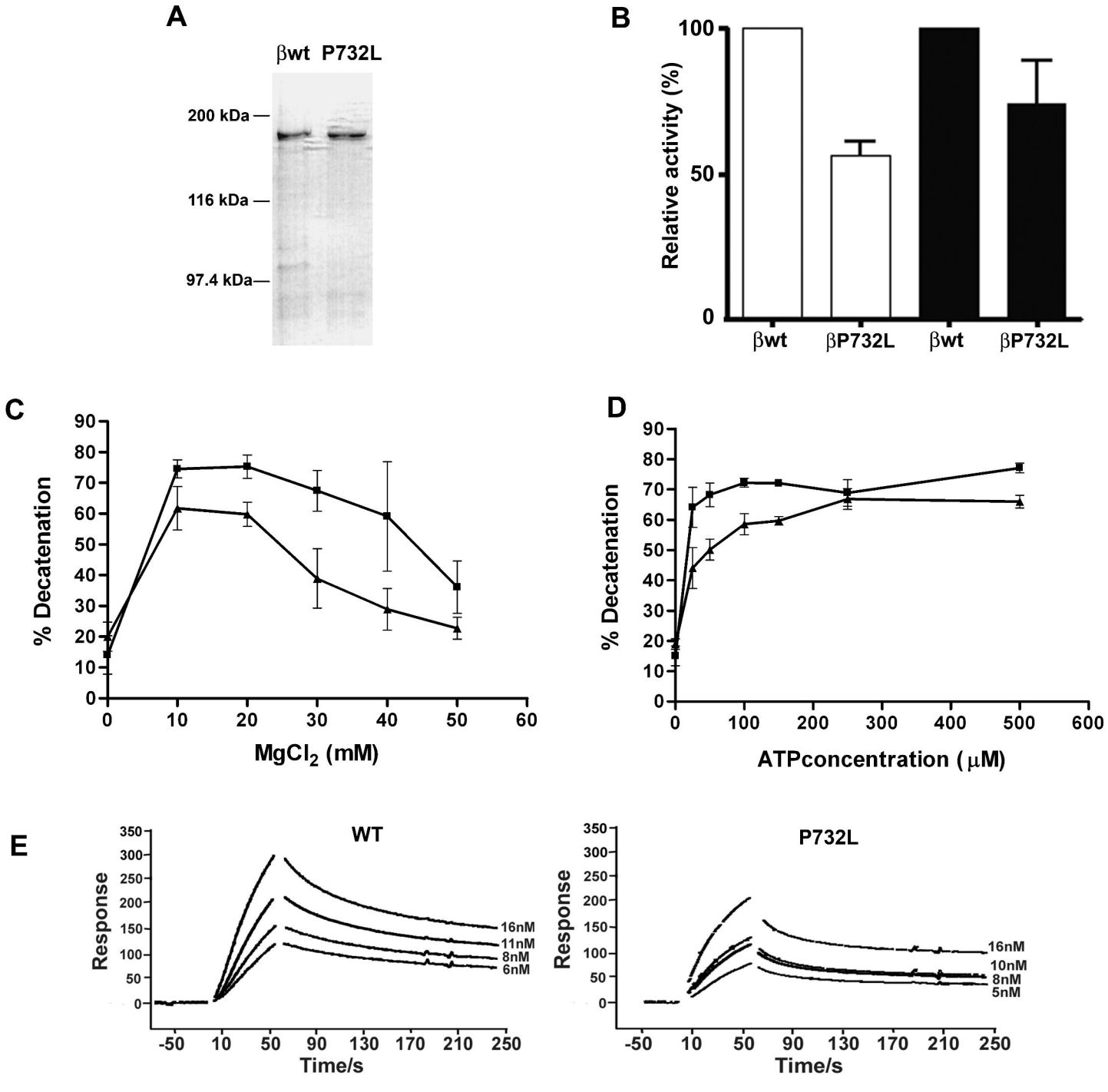
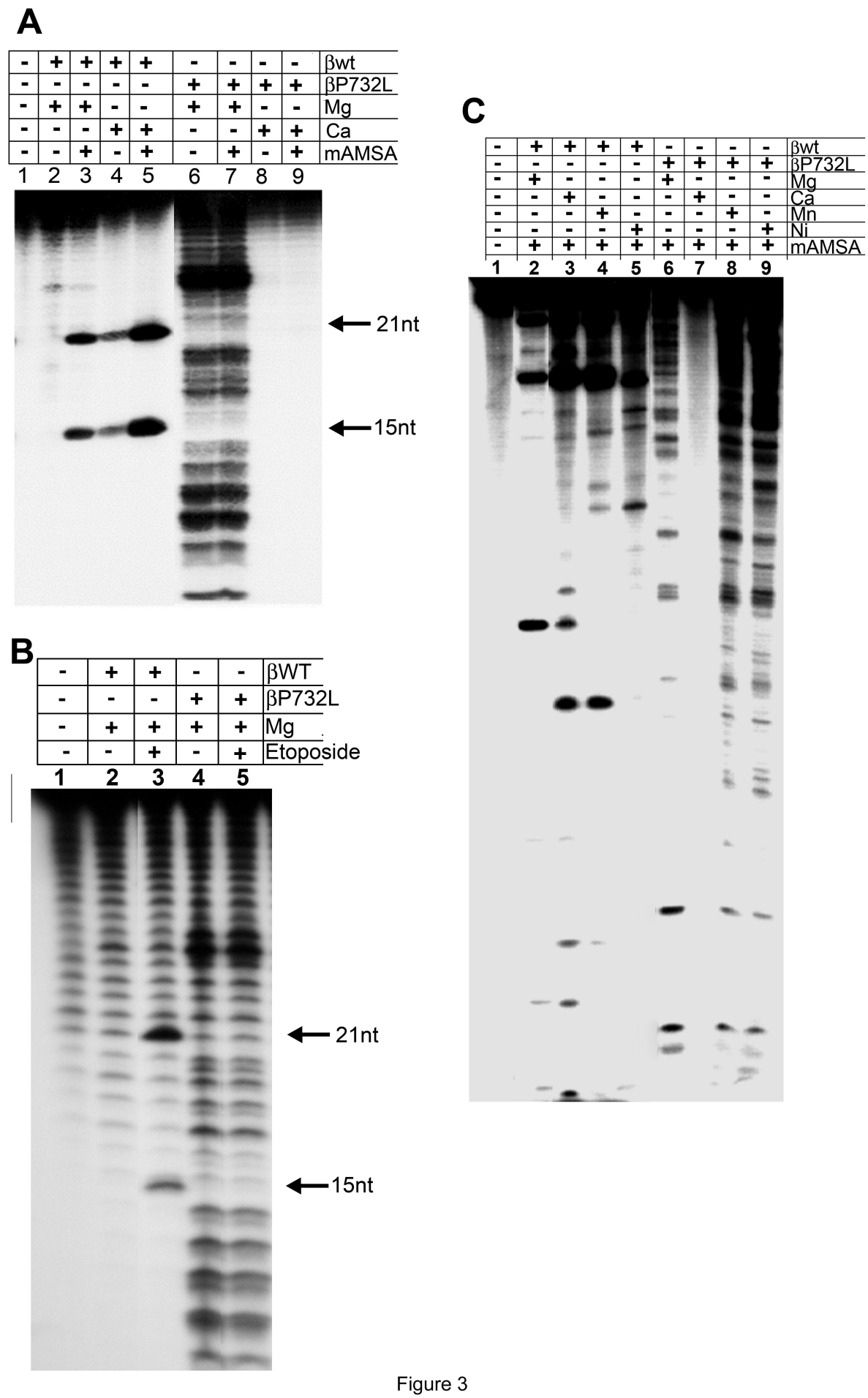


Figure 2


A
- + + + + - - - - βwt
- - - - - + + + + βP732L
- + + - - + + - - Mg
- - - + + - - + + Ca
- - + - + - + - + mAMSA
1 2 3 4 5 6 7 8 9
21nt
15nt
B
- + + - - βWT
- - - + + βP732L
- + + + + Mg
- - + - + Etoposide
1 2 3 4 5
21nt
15nt
C
- + + + + - - - - βwt
- - - - - + + + + βP732L
- + - - - + - - - Mg
- - + - - - + - - Ca
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- - - - + - - - + Ni
- + + + + + + + + mAMSA
1 2 3 4 5 6 7 8 9

Figure 3

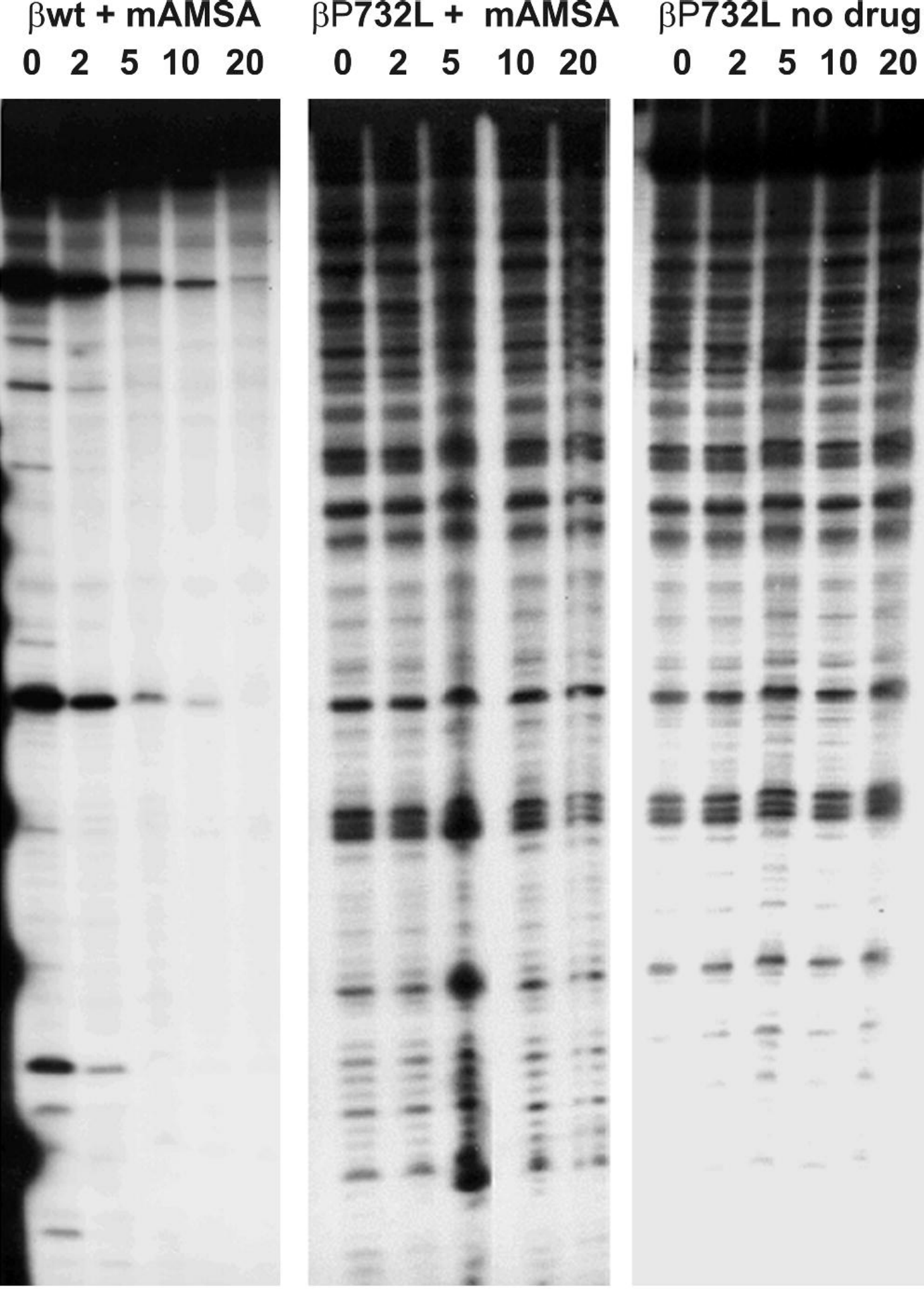
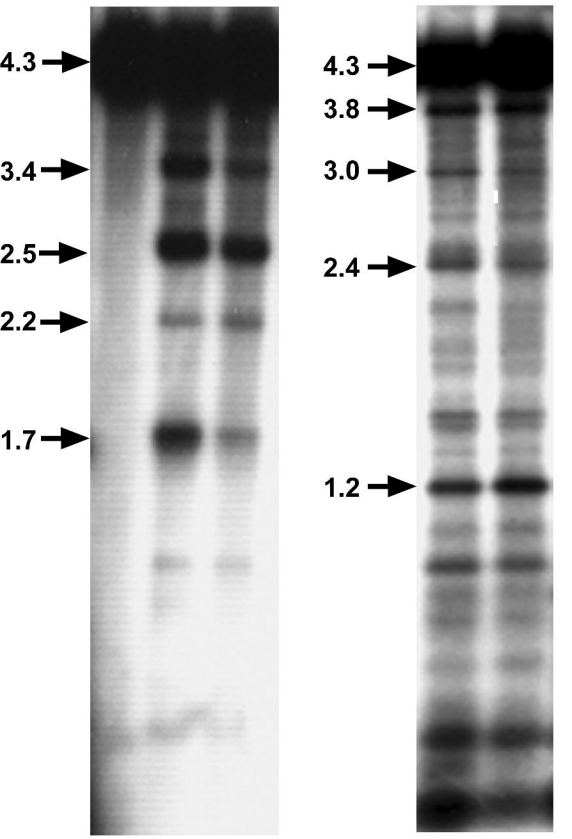

βwt + mAMSA
0 2 5 10 20
βP732L + mAMSA
0 2 5 10 20
βP732L no drug
0 2 5 10 20

Figure 4

A

mAMSA	-	+	+		-	-
Etoposide	-	-	-		+	+
βWT	-	+	-		+	-
βP732L	-	-	+		-	+

1 2 3 4 5



B

-	+	+	+	+	-	-	-	-	βwt
-	-	-	-	-	+	+	+	+	βP732L
-	+	+	-	-	+	+	-	-	Mg
-	-	-	+	+	-	-	+	+	Ca
-	-	+	-	+	-	+	-	+	mAMSA

1 2 3 4 5 6 7 8 9

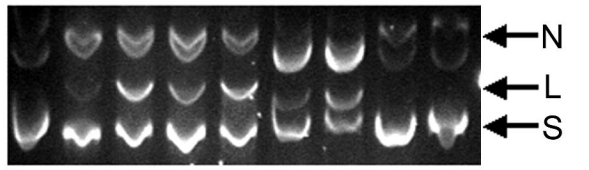
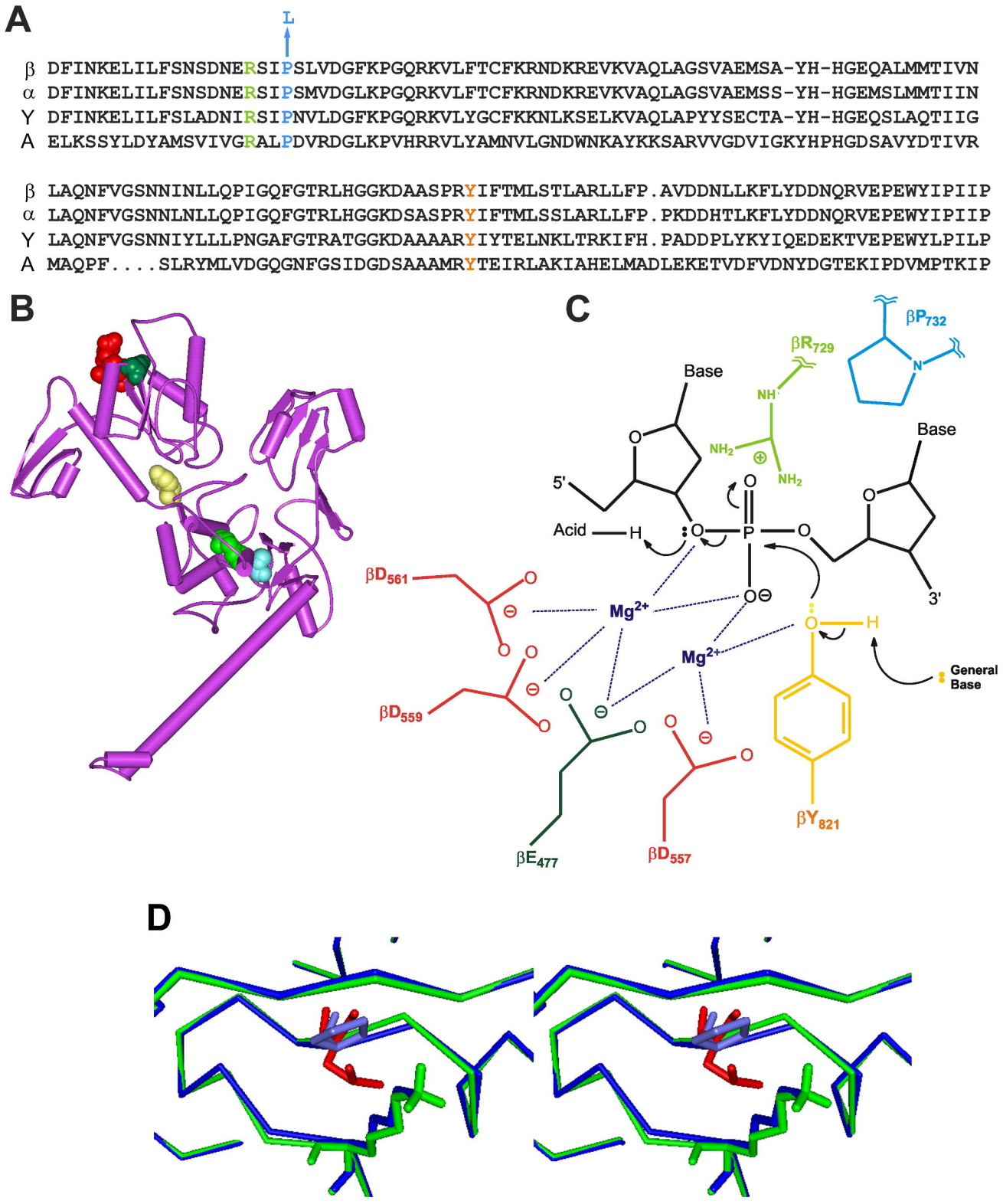


Figure 5


A
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Y DFINKELILFSLADNIRSIPNVLDGFKPGQRKVLYGCFKKNLKSELKVAQLAPYYSECTA-YH-HGEQSLAQTIIG
A ELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVR
β LAQNFVGSNNINLLQPIGQFGTRLHGGKDAASPRYIFTMLSTLARLLFP.AVDDNLLKFLYDDNQRVEPEWYIPIIP
α LAQNFVGSNNLNLLQPIGQFGTRLHGGKDSASPRYIFTMLSSLARLLFP.PKDDHTLKFLYDDNQRVEPEWYIPIIP
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B
C
βP732
βR729
Base
NH
NH2
NH2
Base
5'
Acid
H
O
P
O
3'
βD561
Mg2+
βD559
Mg2+
General Base
βE477
βD557
βY821
D

Fiigure 6