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Activation of mammalian DNA ligase I through phosphorylation by casein kinase II

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Mammalian DNA ligase I has been shown to be a phosphoprotein. Dephosphorylation of purified DNA ligase I causes inactivation, an effect dependent on the presence of the N-terminal region of the protein. Expression of full-length human DNA ligase I in *Escherichia coli* yielded soluble but catalytically inactive enzyme whereas an N-terminally truncated form expressed activity. Incubation of the full-length preparation from *E.coli* with purified casein kinase II (CKII) resulted in phosphorylation of the N-terminal region and was accompanied by activation of the DNA ligase. Of a variety of purified protein kinases tested, only CKII stimulated the activity of calf thymus DNA ligase I. Tryptic phosphopeptide analysis of DNA ligase I revealed that CKII specifically phosphorylated a major peptide also apparently phosphorylated in cells, implying that CKII is a protein kinase acting on DNA ligase I in the cell nucleus. These data suggest that DNA ligase I is negatively regulated by its N-terminal region and that this inhibition can be relieved by post-translational modification.

Key words: casein kinase II/DNA ligase/DNA repair/DNA replication/phosphoserine

Introduction

Mammalian cells contain three distinct ATP-dependent DNA ligases (Tomkinson *et al.*, 1991a). A major activity in the nuclei of actively dividing cells is DNA ligase I, which shows sequence homology to the *cdc*17$^+$ gene product of *Schizosaccharomyces pombe* and the *CDC9* gene product of *Saccharomyces cerevisiae*. Moreover, a cDNA encoding human DNA ligase I can functionally complement a conditionally lethal *cdc9* mutant (Barnes *et al.*, 1990). A human cell line with a mutationally altered, malfunctioning DNA ligase I has been established, and the properties of these cells indicate a role for DNA ligase I both in the joining of Okazaki fragments during DNA replication and in the completion of DNA excision-repair (Barnes *et al.*, 1992). In contrast to DNA ligases II and III, which are present at similar levels independently of the state of cellular proliferation, DNA ligase I activity increases ~10-fold on stimulation of quiescent cells (Soderhall, 1976; Elder and Rossignol, 1990). In this regard it resembles several other replication factors such as DNA polymerase α (Cripps-Wolfman *et al.*, 1989; Nasheuer *et al.*, 1991) and DNA topoisomerase II (Ackerman *et al.*, 1985; Saijo *et al.*, 1990), which are regulated both at the transcriptional and post-translational level.

DNA ligase I is a 102 kDa protein with a 24 kDa N-terminal region which is not required for catalytic activity *in vitro*. This N-terminal region is hydrophilic and very sensitive to proteases *in vitro*, and it confers an apparently asymmetrical structure on the enzyme (Tomkinson *et al.*, 1990). A large C-terminal active fragment is often generated by proteolysis during enzyme purification, but immunoblotting experiments indicate that it does not occur *in vivo* (Lasko *et al.*, 1990a). The role of the N-terminal region of DNA ligase I is unclear, although it contains sequences that might function as a nuclear translocation signal and is rich in acidic residues that might mediate interactions with chromatin. DNA ligase I is apparently part of a large protein complex comprised of several replication factors (Malkas *et al.*, 1990), and the N-terminal region of DNA ligase I could account for such protein-protein interactions. In the present work, we describe the obligatory phosphorylation of the N-terminal region of active DNA ligase I by the protein kinase CKII.

Results

Immunoprecipitation of ^{32}P-labelled DNA ligase I from cell extracts

A rabbit antiserum against homogeneous calf thymus DNA ligase I (Lasko *et al.*, 1990a) was employed for immunopurification of the enzyme from crude extracts of Madin-Darby bovine kidney (MDBK) cells grown in the presence of $^{32}P_i$. Analysis of the immunoprecipitate by SDS-PAGE and autoradiography showed a single radioactive protein band, which was present at the position expected for DNA ligase I (Figure 1A). Addition of purified DNA ligase I to the cell extract before immunoprecipitation quenched the signal and served to identify the radioactively labelled immunopurified protein as DNA ligase I (Figure 1A).

The first step in the joining of DNA strand breaks by an ATP-dependent DNA ligase is the formation of an enzyme-AMP reaction intermediate, with the release of pyrophosphate (Engler and Richardson, 1982). The AMP residue is linked by a phosphoramidate bond to a lysine residue in the protein. In calf thymus cell extracts, DNA ligase I is partly present as a DNA ligase-AMP complex (Soderhall and Lindahl, 1973). Therefore, at least some of the DNA ligase I was expected to have incorporated ^{32}P through adenylylation of the enzyme by [α-^{32}P]ATP formed during metabolic labelling. DNA ligase-AMP complexes

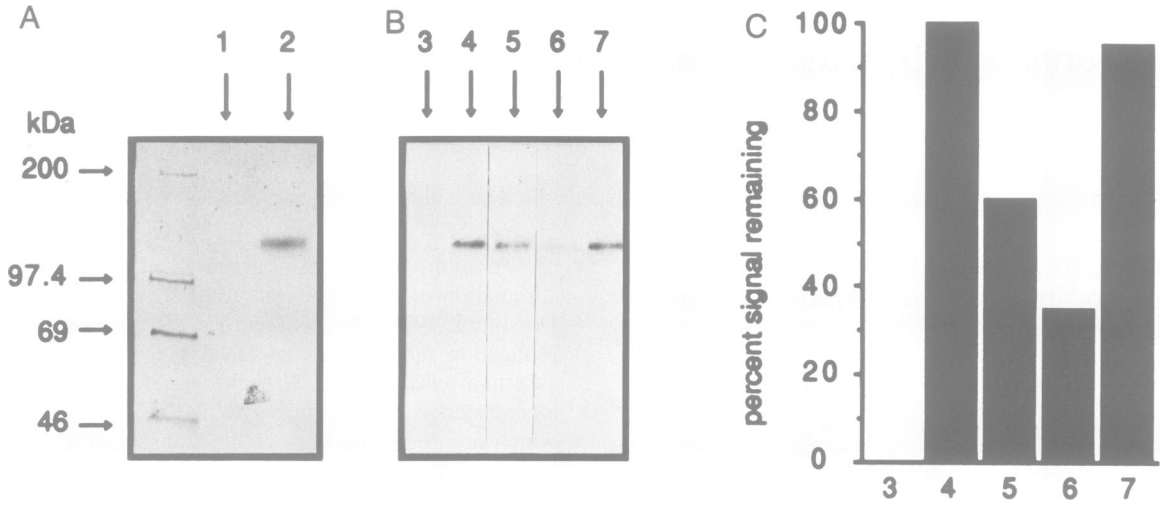

Fig. 1. Phosphorylation of DNA ligase I in ^{32}P-labelled cells. MDBK cells were labelled and lysed, and DNA ligase I was immunoprecipitated with antiserum against homogeneous calf thymus DNA ligase I, or pre-immune serum, and protein A–Sepharose beads. The protein was analysed by SDS–PAGE and autoradiography. (**A**) Lane 1: 10 μg homogeneous calf thymus DNA ligase I was added to the cell lysate prior to immunoprecipitation; lane 2, no addition of non-radioactive DNA ligase I. (**B**) Analysis of the radioactive material in the immunoprecipitate. Lane 3 (pre-immune serum) and lane 4 (immune serum): immunoprecipitated protein was not treated further; lane 5: immunoprecipitate incubated for 10 min in the presence of 10 mM $MgCl_2$ and 0.2 mM PP_i; lane 6, immunoprecipitate treated with PAP (0.1 U); lane 7, as lane 6, but the reaction mixture also contained 100 mM phosphate. (**c**) Densitometry of lanes 3–7.

are readily dissociated by incubation with an excess of pyrophosphate, and such treatment of immunoprecipitates removed 40 ± 5% of the radioactive material from the protein (Figure 1B and C). However, 65 ± 5% of the radioactive material could be removed from the DNA ligase by treatment with potato acid phosphatase (PAP) (Figure 1B and C). The release of this material was prevented by inhibition of the PAP with free phosphate (Figure 1B and C). These data demonstrate that DNA ligase I is a phosphoprotein containing one or several modified amino acid residues susceptible to phosphatase treatment.

Altered migration rate of dephosphorylated DNA ligase I

The purified active 102 kDa DNA ligase I from bovine or human cells migrates anomalously slowly during SDS–PAGE, as if it had a molecular mass of 125–130 kDa (Teraoka and Tsukada, 1982; Tomkinson *et al.*, 1990). Immunoblotting experiments with human tissue culture cell extracts yielded a doublet band of DNA ligase I (Lasko *et al.*, 1990b) corresponding to apparent molecular mass estimates of 125 and 118 kDa. To determine whether the delayed electrophoretic migration could be ascribed to phosphorylation of the enzyme, purified calf thymus DNA ligase I was treated with PAP or bacterial alkaline phosphatase (BAP) in the presence of bovine serum albumin and protease inhibitors. As a control, DNA ligase I was also incubated with PAP and BAP in the presence of phosphate. Dephosphorylation of the enzyme caused a slight but significant increase in its migration rate (Figure 2) corresponding to a decrease in apparent molecular mass from 125 to 118 kDa. Thus, phosphorylation of DNA ligase I provides a partial explanation for the unusually retarded migration rate of the enzyme. However, most of this effect is probably due to the relatively high proline content of the protein (Barnes *et al.*, 1990).

During purification of DNA ligase I from bovine or human cells, part of the protein is degraded by endogenous

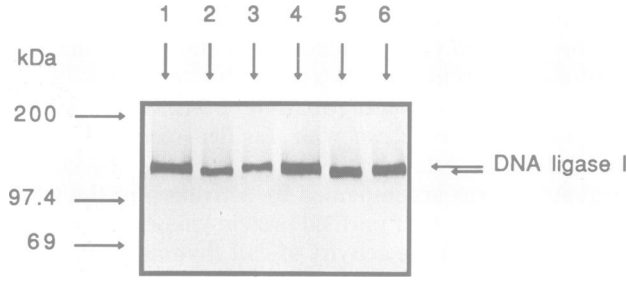

Fig. 2. Altered migration of DNA ligase I during SDS–PAGE after phosphatase treatment. Purified calf thymus DNA ligase I was incubated with PAP (lanes 1–3) or BAP (lane 4–6) and analysed by SDS–PAGE followed by immunoblotting with antiserum against homogeneous calf thymus DNA ligase I (Lasko *et al.*, 1990b) and alkaline phosphatase-conjugated goat anti-rabbit IgG (secondary antibody; Bio-Rad) assayed according to Leary *et al.* (1983). Lanes 1–3 show data with PAP, and lanes 4–6 data with BAP. Lanes 1 and 4: DNA ligase I incubated with phosphatase and 0.1 M phosphate; lanes 2 and 5: DNA ligase I incubated with phosphatase alone; lanes 3 and 6: DNA ligase I incubated in the reaction buffer, without phosphatase.

proteolysis to a 78 kDa active fragment lacking the N-terminal region (Tomkinson *et al.*, 1990). This fragment represents a relatively protease-resistant catalytic domain and was separately purified, free from the 102 kDa form. Its electrophoretic migration rate is only slightly delayed, suggesting a molecular mass of 85 kDa. In contrast to the results with intact DNA ligase I, treatment of this active fragment of the enzyme with PAP or BAP caused no detectable change in its migration rate (data not shown), indicating that the phosphorylated amino acid residues were present largely in the N-terminal region of DNA ligase I.

Inactivation of DNA ligase I by phosphatase treatment

Purified calf thymus DNA ligase I was incubated in appropriate reaction mixtures with PAP or BAP to dephosphorylate the protein. The activity of the enzyme was

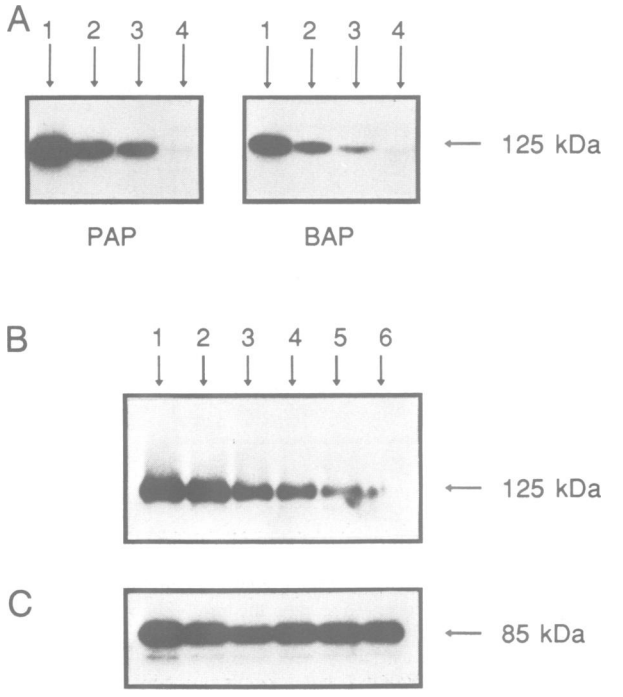

Fig. 3. Inhibition of enzymatic activity of DNA ligase I by phosphatase treatment. Purified calf thymus DNA ligase I (200 μg/ml) was incubated with a phosphatase in the appropriate buffer in a final reaction volume of 50 μl. At appropriate time points 7 μl samples were taken and the reactions were stopped by adding phosphate (to 0.1 M). Enzyme–AMP formation was then monitored following the addition of 1 μl 0.6 M Tris–HCl, pH 8.0, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 500 μg/ml bovine serum albumin, containing 0.5 μCi [α-^{32}P]ATP, in a final volume of 10 μl, and samples were analysed by SDS–PAGE and autoradiography. (**A**) Decreased formation of DNA ligase I–AMP complexes after dephosphorylation of DNA ligase I with increasing amounts of phosphatases. The DNA ligase I was treated with PAP or BAP as indicated. Lane 1: no phosphatase; lane 2: 1 mU phosphatase; lane 3: 10 mU phosphatase; lane 4: 0.1 U phosphatase. (**B**) Kinetics of inactivation of DNA ligase I by treatment with BAP. Lane 1: no phosphatase treatment; lane 2: 5 min incubation with BAP; lane 3: 10 min; lane 4: 25 min; lane 5: 50 min; lane 6: 90 min. (**C**) Treatment of the 78 kDa catalytic domain of DNA ligase I with BAP. Times of incubation as in (B).

then assessed by two different assays, formation of a DNA ligase–AMP complex and ligation of an oligo(dT)·poly(dA) substrate (Tomkinson *et al.*, 1990). Degradation of ATP by the phosphatases was insignificant in these experiments, due to the very rapid formation of a phosphatase-resistant DNA ligase–AMP complex in the presence of ATP (Tomkinson *et al.*, 1991b) and a high ATP concentration in the ligation assay.

The ability of DNA ligase I to form an enzyme–AMP complex was strongly decreased by incubation with PAP or BAP (Figure 3A). Similarly, the DNA ligation activity of the enzyme was obliterated although immunoblotting experiments demonstrated that there was no detectable loss or degradation of the DNA ligase protein (data not shown).

The decrease in activity of the 102 kDa DNA ligase I occurred as a function of time on incubation with BAP (Figure 3B). When the purified C-terminal 78 kDa catalytic domain of DNA ligase I was incubated with BAP under identical conditions, however, no such decrease in activity was observed (Figure 3C). These results show that dephosphorylation of one or several phosphoamino acid residues located in a region outside the catalytic domain of DNA ligase I interfered strongly with the ability of the protein to generate an enzyme–AMP reaction intermediate.

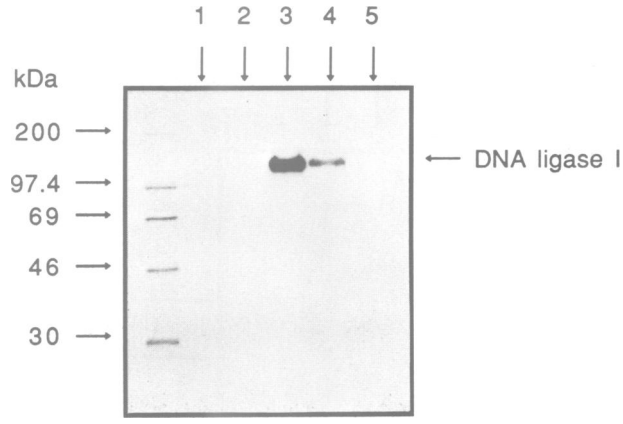

Fig. 4. Phosphorylation of purified DNA ligase I by CKII. Calf thymus DNA ligase I was phosphorylated *in vitro* with CKII in the presence of [γ-^{32}P]ATP and analysed by SDS–PAGE and autoradiography. The complete reaction mixture is shown in lane 3. Lane 1: no DNA ligase I added; lane 2: no CKII added; lane 4: reaction mixture supplemented with 10 mM GTP; lane 5: reaction mixture supplemented with 20 μg/ml heparin.

Phosphorylation of DNA ligase I by protein kinases in vitro

The predicted amino acid sequence of human DNA ligase I contains several putative targets for phosphorylation corresponding to consensus recognition sites for CKII, p34^{cdc2} protein kinase, cyclic AMP-dependent protein kinase (PK-A) and protein kinase C (PK-C). Although the sequence of the similar bovine DNA ligase I has been determined only in part (Barnes *et al.*, 1990), it seems likely that most or all of the putative phosphorylation sites would be present in both the human and bovine proteins. In apparent agreement with these predictions, purified calf thymus DNA ligase I was found to be a substrate *in vitro* for ATP-dependent phosphorylation by all these protein kinases. This could reflect generation of acceptor sites by dephosphorylation of DNA ligase I *in vivo* or during enzyme purification, and lack of phosphorylation *in vivo* by some of the kinases. The data obtained by CKII treatment are shown in Figure 4. The phosphorylation of DNA ligase I by CKII, employing [γ-^{32}P]ATP, was inhibited by heparin, a known inhibitor of CKII (Hathaway *et al.*, 1980). Moreover, this protein kinase can employ GTP as a cofactor almost as efficiently as ATP, and addition of GTP to the reaction mixture suppressed the protein phosphorylation with [γ-^{32}P]ATP. The incorporation of phosphate catalysed by CKII corresponded to 0.5–0.6 mol P per mol DNA ligase I.

In contrast to these results with the intact 102 kDa DNA ligase I, the purified 78 kDa catalytic domain of DNA ligase I was a poor substrate for CKII, and incorporation of phosphate into this domain was $<10\%$ of that observed with the 102 kDa protein. These data indicate that the major site of phosphorylation of DNA ligase I by CKII *in vitro* is present in the N-terminal region of the DNA ligase protein.

Increased DNA ligase I activity after phosphorylation by CKII

Calf thymus DNA ligase I was incubated with the various protein kinases in the presence of ATP under conditions

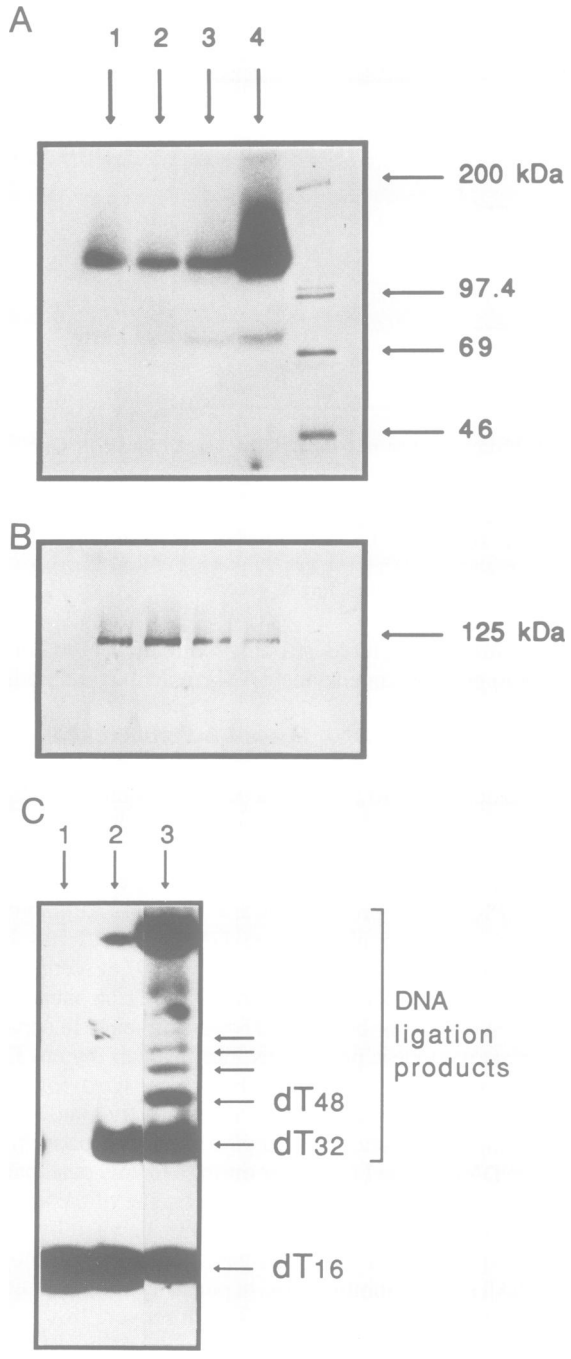

Fig. 5. Increased enzymatic activity of calf thymus DNA ligase I phosphorylated with CKII. DNA ligase I was incubated with CKII and GTP as follows (A and B): lane 1: without CKII; lane 2: without CKII but with heparin; lane 3: with both CKII and heparin; lane 4: with CKII. (**A**) Enzyme–AMP formation. One-half of each reaction mixture was incubated with [α-^{32}P]ATP under standard adenylation conditions, and analysed by SDS–PAGE and autoradiography. (**B**) Immunoblotting. One-half of each reaction mixture was analysed by SDS–PAGE, followed by immunoblotting as in Figure 2. (**C**) Joining of strand breaks. DNA ligase I was incubated with [5′-^{32}P](dT)$_{16}$·poly(dA) in the presence of ATP (10^{-4} M). Ligation products were analysed by 20% polyacrylamide gel electrophoresis in the presence of 8 M urea. Lane 1: no DNA ligase; lane 2: DNA ligase I without CKII; lane 3: DNA ligase I and CKII.

appropriate for protein phosphorylation. The reaction mixtures were then further incubated with a non-inactivating rabbit antiserum against the purified DNA ligase, and the

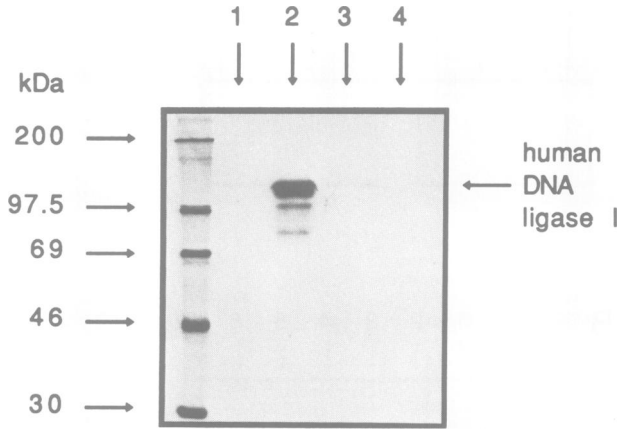

Fig. 6. Activation of human DNA ligase I expressed in *E.coli* by CKII phosphorylation. Crude cell extracts of *E.coli* containing the human DNA ligase I cDNA expressed in pBluescript were incubated with CKII, and then assayed for DNA ligase I–AMP formation by incubation with [α-^{32}P]ATP, followed by SDS–PAGE and autoradiography. The complete reaction mixture is shown in lane 2. Lane 1: no CKII added; lane 3: reaction mixture supplemented with heparin (10 μg/ml); lane 4: *E.coli* cell extract containing only the expression vector without DNA ligase I cDNA.

DNA ligase I–antibody complexes immunoprecipitated with protein A–Sepharose beads (Lasko *et al.*, 1990a). The beads were treated with pyrophosphate to disrupt enzyme–AMP complexes and then washed to remove the pyrophosphate. The immunoprecipitated DNA ligase I was assayed, both for formation of enzyme–AMP complexes and ligation of a [5′-^{32}P]oligo(dT)·poly(dA) substrate. Incubation of the purified calf thymus DNA ligase I with CKII caused an ~5-fold increase in activity, as measured by either assay. In contrast, no increase in DNA ligase I activity was detected after phosphorylation with p34^{cdc2} protein kinase, PK-A or PK-C (data not shown).

Since CKII can employ GTP as phosphate donor, whereas DNA ligase I is unable to use GTP as cofactor in the ligation reaction, a simplified protocol was designed for further investigations of the effect of CKII phosphorylation. DNA ligase I was phosphorylated by CKII in the presence of non-radioactive GTP, and directly assayed for ability to form an enzyme–AMP complex in the presence of [α-^{32}P]ATP. In this way, a 6-fold stimulation of the activity of calf thymus DNA ligase I was observed after CKII phosphorylation (Figure 5A). These experiments were carried out at low CKII concentrations, so DNA ligase I is a good substrate for this protein kinase. A similar increase in DNA ligase activity after phosphorylation was observed when an oligo(dT)·poly(dA) polymer was employed in ligation assays (Figure 5C). The increase in activity was prevented by including heparin in the reaction mixture, although heparin at this concentration did not inhibit DNA ligase I (Figure 5A). Parallel immunoblotting data showed that the amount of DNA ligase I protein did not vary significantly between the different reaction mixtures (Figure 5B).

Activation of human DNA ligase I expressed in E.coli by CKII phosphorylation

A cDNA encoding the C-terminal catalytic domain of human DNA ligase I functionally complements the *E.coli* conditionally lethal *lig*-251 mutant and permits bacterial growth at the restrictive temperature of 42°C (Kodama

et al., 1991). In contrast, a cDNA encoding the intact 102 kDa human DNA ligase I fails to complement the *E.coli lig* mutant, although the human protein is expressed in the bacteria. Furthermore, when these two forms of human DNA ligase I were extracted from *E.coli* and assayed for their ability to generate an enzyme–AMP complex, the isolated catalytic domain was active whereas no activity was detected of the full-length enzyme (Kodama *et al.*, 1991). Results obtained after transfection of a *S.cerevisiae cdc9* mutant are different, in that cDNAs encoding either the full-length human DNA ligase I or the catalytic domain alone both complemented the *cdc9* phenotype (Barnes *et al.*, 1990). One possible explanation of these data is that functional expression of the full-length DNA ligase I may only occur after phosphorylation by a protein kinase, such as CKII, which is ubiquitous in eukaryotic cells but not present in bacteria.

In order to determine whether human DNA ligase I overexpressed in *E.coli* could be activated by phosphorylation with CKII, the ability of the soluble human enzyme to generate a DNA ligase–AMP reaction intermediate in *E.coli* extracts was assessed. In agreement with previous observations, full-length human DNA ligase I was inactive (Kodama *et al.*, 1991). However, the enzyme was found to be converted to an active form by CKII (Figure 6). Activation of DNA ligase I was prevented by inhibition of CKII with heparin (Figure 6). Thus, phosphorylation by CKII is both necessary and sufficient for enzymatic activity of full-length DNA ligase I.

Formation of phosphoserine in vivo and in vitro

DNA ligase I was immunoprecipitated from cells metabolically labelled with $^{32}P_i$ and hydrolysed with 6 N HCl at 110°C to liberate free phosphoamino acids. The phosphoamino acids, together with appropriate reference compounds, were separated by electrophoresis. Phosphoserine was shown to be present by autoradiography, whereas phosphothreonine and phosphotyrosine were not detected (Figure 7). A trace of phosphothreonine could be observed after prolonged exposure of autoradiographs.

Similar phosphoamino acid analysis of calf thymus DNA ligase I phosphorylated *in vitro* by several purified protein kinases showed that phosphoserine was generated largely or exclusively by CKII (Figure 7), $p34^{cdc2}$ and PK-A. In contrast, PK-C phosphorylated DNA ligase I on threonine.

DNA ligase I phosphorylation sites in vivo and in vitro

Immunoprecipitated DNA ligase I from metabolically ^{32}P-labelled MDBK cells, or calf thymus DNA ligase I phosphorylated *in vitro* by one of several protein kinases in the presence of [γ-^{32}P]ATP, were extensively digested with trypsin. The resulting phosphopeptides were resolved by two-dimensional separation on thin layer plates, involving electrophoresis and ascending chromatography, and detected by autoradiography. The DNA ligase I labelled *in vivo* generated about six distinct, strongly labelled peptides and a few weakly labelled peptides (Figure 8). Some of the latter may have been due to partial trypsin cleavage at double basic residues. One of the prevalent phosphopeptides from the *in vivo* labelled material co-migrated with the major, apparently hydrophilic peptide phosphorylated by CKII *in vitro*, as estimated from analysis of a mixture of the two samples (Figure 8). The *in vitro* phosphorylation of calf thymus DNA ligase I by CKII yielded 0.55 mol phosphate incorporated per mol protein. This is consistent with the observation that the isolated DNA ligase I is partly phosphorylated. The data indicate that DNA ligase I is modified at a distinct serine residue by CKII *in vivo*. Two other tryptic peptides (indicated by small arrows in Figure 8b) from metabolically labelled DNA ligase I showed the same migration pattern as the two major phosphopeptides observed after treatment of the purified enzyme with $p34^{cdc2}$ *in vitro* (data not shown). It seems likely that DNA ligase I is phosphorylated *in vivo* by at least two different protein kinases. However, we have only observed a functional effect in the case of CKII phosphorylation, in that the enzymatic activity of DNA ligase I appears to be directly dependent on such post-translational modification.

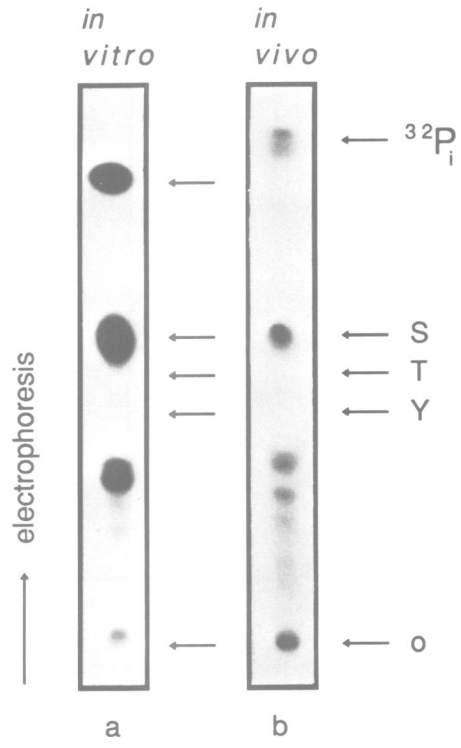

Fig. 7. Phosphoamino acid analysis of bovine DNA ligase I phosphorylated *in vivo* and *in vitro*. DNA ligase I was hydrolysed and mixed with non-radioactive markers; phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). Phosphoamino acids were separated by high voltage electrophoresis on TLC plates and detected by autoradiography. Non-radioactive reference compounds were visualized by ninhydrin staining.

Discussion

The serine/threonine protein kinase, CKII, is present at a high level in the nuclei and cytoplasm of proliferating eukaryotic cells (Krebs *et al.*, 1988; Krek *et al.*, 1992). The physiological roles of CKII are largely unknown, but the similar catalytic subunits of the enzyme show strong evolutionary conservation. In *S.cerevisiae* disruption of both genes encoding CKII catalytic subunits is a lethal event (Padmanabha *et al.*, 1990). Modulation of the functions of several transcription factors, oncogene products and DNA replication factors by CKII phosphorylation has been described. Thus, modification of the c-*myb* transcription factor by CKII phosphorylation causes down-regulation by

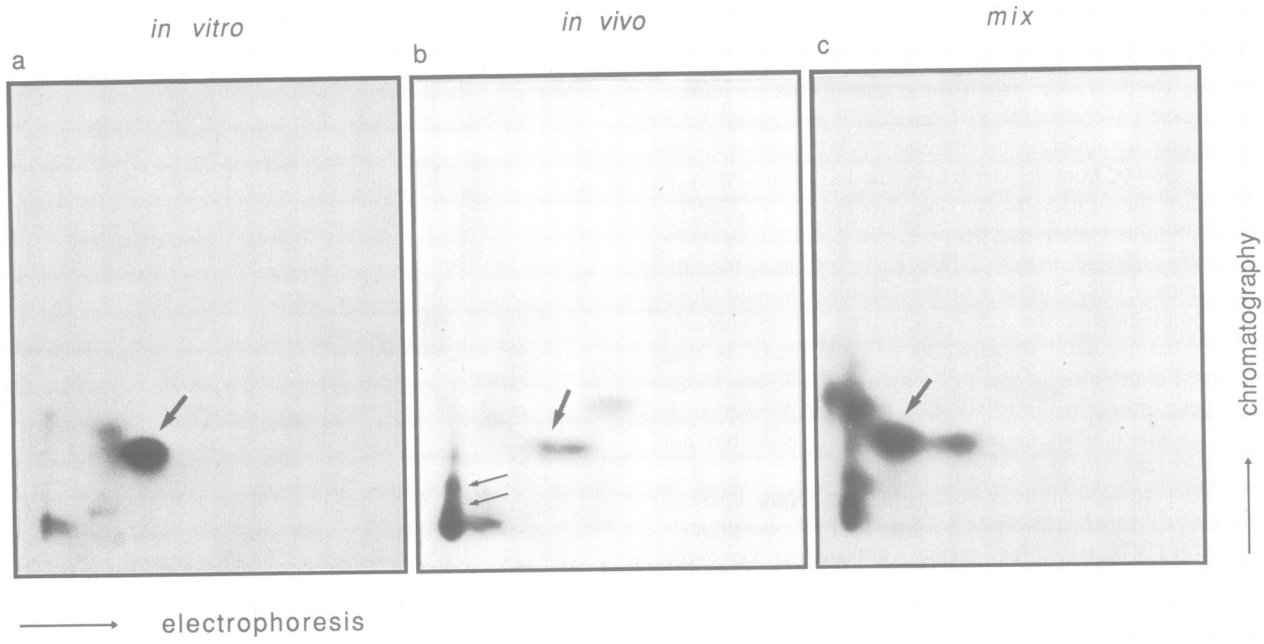

Fig. 8. Two-dimensional analysis of tryptic phosphopeptides of bovine DNA ligase I after phosphorylation *in vitro* and *in vivo*. DNA ligase I ^{32}P-labelled *in vitro* (**a**), *in vivo* (**b**) or a mixture of both (**c**) was digested with trypsin and peptides were separated by high voltage electrophoresis followed by chromatography. Radioactive peptides were visualized by autoradiography. The large arrow indicates a major peptide obtained after both *in vitro* labelling with CKII and *in vivo* labelling. Small arrows indicate peptides which comigrate with the two major peptides phosphorylated by p34^{cdc2} *in vitro*.

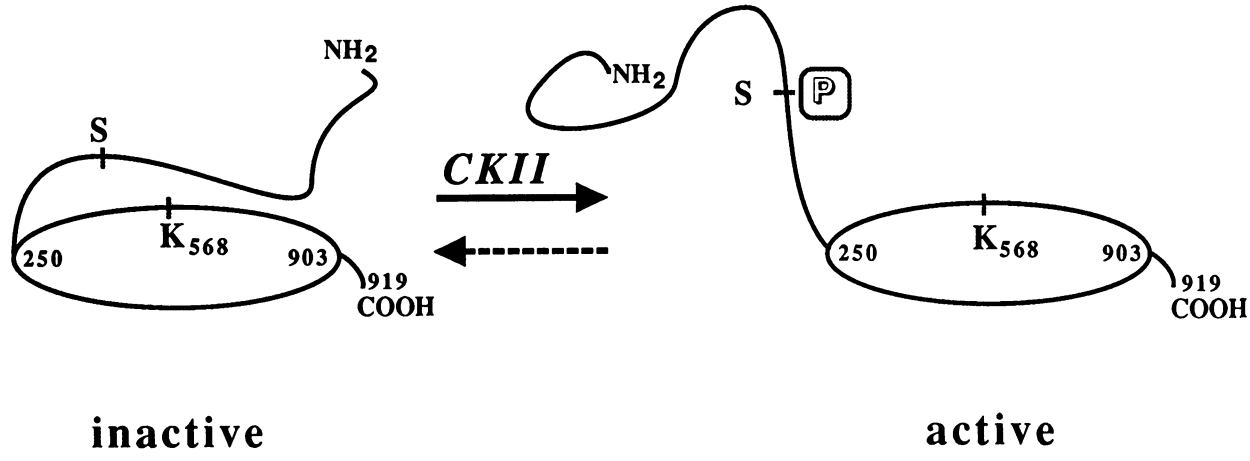

Fig. 9. Activation of mammalian DNA ligase I by CKII phosphorylation. The primary translation product of DNA ligase I is enzymatically inactive, because the N-terminal region of the enzyme interferes with its ability to generate a reaction intermediate by adenylylation at residue Lys568 in the catalytic domain (contained within residues 250–903; Kodama *et al.*, 1991). Post-translational modification of DNA ligase I by phosphorylation of a serine residue in the N-terminal region alleviates the inhibitory effect, presumably by inducing a conformational change in the protein.

inhibiting DNA binding (Luscher *et al.*, 1990), whereas phosphorylation of the serum response factor at multiple sites in the N-terminal region increases its rate of association with DNA (Marais *et al.*, 1992; Janknecht *et al.*, 1992). With regard to DNA replication enzymes, a clear difference in activity due to CKII-catalysed phosphorylation has been demonstrated for DNA topoisomerase II (Ackerman *et al.*, 1985, 1988; Saijo *et al.*, 1990). The topoisomerase occurs as a phosphoprotein in proliferating *Drosophila* and mouse cells, with one or several serine residues apparently modified by CKII, and the enzyme exhibits a large decrease in activity after phosphatase treatment. However, the mechanism for stimulation of DNA topoisomerase II activity by CKII phosphorylation remains to be established.

In the present work, mammalian DNA ligase I has been shown to exist as a phosphoprotein in actively growing cells, and peptide mapping experiments indicated that CKII is partly responsible for the modification of the DNA ligase *in vivo*. Several reagent protein kinases were investigated and observed to phosphorylate purified DNA ligase I *in vitro*, but CKII was the only one which affected the DNA ligation activity of the enzyme. Moreover, CKII treatment activated the full-length but inactive form of DNA ligase I expressed in *E.coli*. These data indicate that phosphorylation of DNA ligase I by CKII is required for DNA ligase activity. The first step in the DNA ligase I reaction mechanism, formation of an enzyme–AMP intermediate, is modulated by CKII phosphorylation. Therefore, the result of phosphorylation

by CKII is not restricted to a change in the affinity of DNA ligase I for its DNA substrate; interaction with the ATP cofactor is directly affected. A model postulating a conformational change after phosphorylation is depicted in Figure 9.

The N-terminal region of DNA ligase I, which contains the CKII phosphorylation site(s), exhibits seven S−−E motifs, as deduced from the human DNA ligase I cDNA sequence, which are possible substrates for CKII (Kemp and Pearson, 1990). Most of the putative CKII phosphorylation sites in DNA ligase I are clustered in the N-terminal region of the protein. It is not yet known which of the seven serine residues is the preferred site. However, two of the sequences have the properties of 'strong' CKII phosphorylation sites, S_{66}EGEEEDE and S_{141}EDEDRE, and one of these is the most likely candidate.

Calf thymus DNA ligase I, purified to homogeneity by conventional procedures, was inactivated by dephosphorylation, whereas the activity was increased ~5-fold by CKII phosphorylation *in vitro*. These data indicate that the enzyme is isolated in a partly phosphorylated form, as a mixture of active and inactive protein molecules. Detailed kinetic investigations of V_{max} and other biochemical parameters of the homogeneous enzyme (Teraoka and Tsukada, 1982), therefore, may require reinvestigation with the fully phosphorylated form of the enzyme. It is not known to what extent DNA ligase I is accidentally dephosphorylated during purification by phosphatases present in crude enzyme fractions. However, it is noteworthy that enzyme purification is conventionally performed in phosphate buffer, which would minimize phosphatase activity. Repeated observations by immunoblotting of a doublet band of DNA ligase I extracted from various human cell lines (Lasko *et al.*, 1990b) also suggest that both the phosphorylated and unphosphorylated forms may be present *in vivo*. The DNA ligase I polypeptide has a relatively long half-life, 7 h, in proliferating MDBK cells (Lasko *et al.*, 1990a), so regulation of the enzyme activity at the transcriptional and translational levels might only be effective over long time periods. Activation and deactivation of the enzyme by CKII phosphorylation versus phosphatase action would offer a more rapid and versatile mode of regulation of the level of DNA ligase I activity during the cell cycle or in response to DNA damaging agents. The phosphorylation pattern of DNA ligase I in human cells exhibiting impaired DNA ligation (Barnes *et al.*, 1992) remains to be investigated. Interestingly, expression of the cDNA for the regulatory β subunit of CKII in DNA repair-deficient xeroderma pigmentosum cells confers partial resistance to ultraviolet light (Teitz *et al.*, 1990), but this may be due to prolonging the G_2 phase of the cell cycle, not directly to activation of a DNA ligase.

The *in vivo* phosphorylation data indicated that DNA ligase I is a substrate for other protein kinases, in addition to CKII. Similar observations have been made for several other nuclear phosphoproteins (Krebs *et al.*, 1988). While we could not relate the additional phosphorylation to functional effects, it is relevant to note that we have only examined the catalytic activity of the enzyme in the present work. Thus, any effect of DNA ligase I phosphorylation on the intracellular distribution of the enzyme, such as the translocation from cytoplasm to nucleus or recruitment into DNA replication complexes, would not have been detected.

Conversion of certain protooncogenes to oncogenes, such as c-*myb* to v-*myb*, has been associated with deletion or truncation of a CKII phosphorylation site preventing down-regulation (Luscher *et al.*, 1990). Deletion of the N-terminal region of DNA ligase I, or alteration of its main CKII phosphorylation site, may similarly lead to the expression of a high level of unregulated DNA ligase I activity in cells. The phenotypic effects of such overexpression could provide further insights into the role of this enzyme in mammalian cells.

Materials and methods

Reagent enzymes

DNA ligase I was purified to apparent homogeneity from calf thymus as described (Tomkinson *et al.*, 1990). The enzyme preparation had a specific activity of 2000 U (Tomkinson *et al.*, 1991a) per mg protein. The 78 kDa catalytic domain of calf thymus DNA ligase I was recovered after Mono-Q chromatography of the enzyme (Tomkinson *et al.*, 1990) as an early-eluting activity.

CKII (~50% pure) was isolated from rabbit skeletal muscle by chromatography on phosphocellulose, heparin−agarose, a peptide affinity column and Mono-Q, as described by Woodgett (1991). The CKII preparation had a specific activity of 400 U/mg against casein, and all detectable activity was inhibited by 200 ng/ml heparin. Protein kinase $p34^{cdc2}$ from starfish oocytes, purified according to Labbé *et al.* (1989), was a gift from M.Dorée. PK-C from bovine brain (~300 U/ml), a mixture of the α, β and γ isotypes (Marais and Parker, 1989), was a gift from P.J.Parker. The catalytic subunit of PK-A from bovine heart was purchased from Sigma.

PAP was purchased from Boehringer Mannheim and dialysed overnight against 100 mM NaCl, 20 mM MES (2-[*N*-morpholino]ethane sulfonic acid)−NaOH, pH 6.5, 1 mM $MgCl_2$ before use. *E.coli* alkaline phosphatase, type III, was purchased from Sigma and further purified from traces of protease activity as follows. The enzyme (3.5 mg/ml) was dialysed overnight against 50 mM Tris−HCl, pH 8.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$. The enzyme solution was then heated at 90°C for 10 min, chilled to 0°C and incubated at 22°C for 6 h. After centrifugation, the supernatant was dialysed overnight at 4°C against 10 mM Tris−HCl, pH 8.0, and applied to a small DEAE−Sepharose (Pharmacia) column equilibrated with the same buffer. After washing, the phosphatase was eluted with 0.3 M NaCl, 10 mM Tris−HCl, pH 8.0, and frozen in aliquots.

Trypsin (sequencing grade) was from Boehringer Mannheim.

Phosphorylation and dephosphophorylation of DNA ligase I in vitro

Calf thymus DNA ligase I (1−10 pmol) was phosphorylated with CKII (10 mU unless otherwise stated) in 50 mM NaCl, 50 mM Tris−HCl, pH 7.5, and 10 mM $MgCl_2$ in the presence of either 100 μM GTP, 100 μM ATP or 10 μCi [γ-^{32}P]ATP (10 Ci/mmol, Amersham) in a reaction volume of 10 μl for 30 min at 25°C. In assays for ^{32}P incorporation, reactions were stopped by heating with SDS sample buffer for 10 min at 90°C, followed by SDS−PAGE and autoradiography. In all experiments, gel electrophoresis was performed essentially as described by Laemmli (1970).

Phosphorylation with $p34^{cdc2}$ and the catalytic subunit of PK-A were performed (with ATP as cofactor) under the same conditions, except that the reaction mixture also contained 1 mM dithiothreitol. Reaction mixtures with PK-C contained, in addition to the above components, 0.1 mM $CaCl_2$, 33 μg/ml phosphatidylserine, and 0.2 μg/ml TPA (12-*O*-tetradecanoyl-phorbol-13-acetate).

Dephosphorylation of calf thymus DNA ligase I (1 pmol per reaction mixture) was performed with BAP (0.1 U unless otherwise stated) in 20 μl of 50 mM Tris−HCl, pH 8.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, a mixture of six protease inhibitors (27 μg/ml aprotinin, 0.17 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml each of leupeptin, pepstatin, chymostatin and tosyl-phenyl-chloromethyl-ketone), 1 mg/ml bovine serum albumin and 10% glycerol for 30 min at 25°C. Dephosphorylation with PAP was performed similarly, except that reaction mixtures contained 20 mM MES−NaOH, pH 6.5, 100 mM NaCl instead of Tris−HCl and $ZnCl_2$.

DNA ligase assays

Formation of DNA ligase I−AMP complexes was measured in a reaction mixture (10 μl) containing 60 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$,

5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 2 μM ATP containing 0.5 μCi [α-^{32}P]ATP (5000 Ci/mmol) and a limiting amount of DNA ligase I. The DNA ligase I was either in solution or attached to affinity-purified DNA ligase I antibodies bound to protein A–Sepharose beads (Lasko *et al.*, 1990b). Incubations were at 25°C for 10 min. SDS sample buffer was added and reaction mixtures were heated at 90°C for 10 min and analysed by SDS–PAGE. Adenylylated DNA ligase I was detected by autoradiography.

Joining of DNA strand breaks by DNA ligase I was measured by the conversion of the oligonucleotide in [5′-^{32}P](dT)$_{16}$·poly(dA) to (dT)$_{32}$ and higher oligomers, using incubation conditions (Tomkinson *et al.*, 1990) and analysis on denaturing polyacrylamide gels (Hardy *et al.*, 1991) as described.

Phosphorylation of DNA ligase I in vivo

Madin–Darby bovine kidney cells [Madin and Darby (1958); American Type Culture Collection CCL 22] were obtained from Flow Laboratories. A bovine cell line was chosen because antibodies against calf thymus DNA ligase I were employed in immunoprecipitation experiments. For metabolic ^{32}P-labelling, MDBK cells were grown at 37°C in monolayer culture in Eagle's minimal medium (E4) supplemented with 10% fetal bovine serum. 8×10^5 cells were grown for 36 h and then supplemented with carrier-free $^{32}P_i$ (0.8 mCi in 1 ml) for 3 h. At the end of the labelling period, the cells were washed in ice-cold phosphate-buffered saline and lysed *in situ* by incubation for 30 min at 0°C in lysis buffer (20 mM sodium phosphate, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA) supplemented with six different protease inhibitors, as above. The lysate was centrifuged and the supernatant used for immunoprecipitation (Harlow and Lane, 1988). The extract was precleared initially using preimmune serum. The DNA ligase I was then immunoprecipitated with polyclonal rabbit antibodies against the enzyme (Lasko *et al.*, 1990a). Immunocomplexes were collected with protein A–Sepharose beads (Pharmacia) and washed four times with lysis buffer. For direct analysis, the beads were incubated in SDS sample buffer for 10 min at 90°C and removed by centrifugation, and the supernatant was then used for SDS–PAGE. In some experiments, immunoprecipitates were washed with phosphatase buffer and incubated at 25°C for 30 min with the relevant phosphatase, as indicated. After treatment, the beads were washed again with lysis buffer, incubated in SDS sample buffer for 10 min at 90°C and centrifuged, and the supernatants were analysed by SDS–PAGE and autoradiography.

For two-dimensional analysis of tryptic peptides of DNA ligase I phosphorylated *in vivo*, the procedure for radioactive labelling of the cells was slightly different. Cells at ~70% confluence were pre-incubated for 2 h at 37°C in phosphate-free E4 medium containing 10% dialysed fetal bovine serum. The cells were scraped from the flasks, transferred to microtubes and incubated for 3 h in 1 ml of the same medium containing 0.8 mCi $^{32}P_i$. The cells were then washed with cold PBS and lysed in the lysis buffer. Immunoprecipitations were performed without the preclearing step. After incubation with Mg^{2+} and pyrophosphate to remove DNA ligase–AMP complexes, the immunoprecipitate was subjected to SDS–PAGE and then electrophoretically transferred onto an Immobilon-P membrane (Millipore) for autoradiography.

Overexpression of human DNA ligase I in E.coli

Full-length human DNA ligase I cDNA (Barnes *et al.*, 1990) in the pBluescript SK$^-$ vector (Stratagene) was expressed in the temperature-sensitive *E.coli lig*-251 mutant (Dermody *et al.*, 1979) as described by Kodama *et al.* (1991). 1 ml bacterial cultures were grown overnight at 30°C, and the cells were collected and disrupted by sonication in 0.7 ml 0.1 M NaCl, 0.1 M Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM sodium pyrophosphate. After removal of debris by centrifugation, 5 μl supernatant was incubated with 10 mU CKII, 100 μM GTP, 0.5 μCi [α-^{32}P]ATP (5000 Ci/mmol), and six protease inhibitors (as above) in a final volume of 10 μl for 30 min at 25°C. Reaction products were analysed by SDS–PAGE and autoradiography.

Phosphopeptide mapping

Phosphorylated ^{32}P-labelled DNA ligase I was purified by SDS–PAGE, electrophoretically transferred onto an Immobilon-P membrane (Millipore) and visualized by autoradiography. The radioactive band corresponding to the phosphorylated DNA ligase I was cut out and the piece of membrane incubated in 0.5% polyvinylpyrrolidone in 100 mM acetic acid at 37°C for 30 min, then washed with water followed by 50 mM ammonium bicarbonate. The protein was digested by incubating the piece of membrane in 200 μl of 50 mM ammonium bicarbonate containing 10 μg trypsin for 2 h at 37°C, followed by addition of another 10 μg of trypsin and a second incubation overnight at 37°C. Tryptic peptides released from the membrane were lyophilized and washed twice with 100 μl of water. The lyophilized peptides were then oxidized at 0°C for 60 min in 100 μl of performic acid, lyophilized, washed twice with 100 μl of water and lyophilized again, then dissolved in electrophoresis buffer, pH 1.9, and analysed by two-dimensional separation on TLC plates (20 cm × 14 cm polyester silica gel, 250 μm thick, Macherey–Nagel). The first dimension involved electrophoresis at high voltage, performed with an Isothermal Controlled Electrophoresis System (EC 1001) at 750 V, for 40 min in 2.2% formic acid, 7.8% acetic acid, pH 1.9. The second dimension employed ascendant chromatography in 37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid. Phosphopeptides were visualized by autoradiography of the TLC plate (Boyle *et al.*, 1991).

Phosphoamino acid analysis

After transfer, an Immobilon-P membrane piece containing ^{32}P-labelled DNA ligase I was incubated at 110°C for 1 h in 200 μl 6 N HCl under nitrogen. The amino acids were lyophilized and washed several times with water. The ^{32}P-phosphoamino acids were mixed with non-radioactive phosphotyrosine, phosphothreonine and phosphoserine (Sigma, 1 μg of each) and separated by high voltage electrophoresis on TLC plates in 5% acetic acid, 0.5% pyridine, pH 3.5, at 750 V for 20 min. Radioactive phosphoamino acids were detected by autoradiography and the non-radioactive phosphoamino acids by staining with ninhydrin.

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