Cell Cycle-dependent Phosphorylation of Human DNA Ligase I at the Cyclin-dependent Kinase Sites*

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Giovanni Ferrari‡§, Rossella Rossi‡§, Daniele Arosio¶, Alessandro Vindigni¶,
Giuseppe Biamonti‡, and Alessandra Montecucco‡

From the Istituto di Genetica Molecolare, Consiglio Nazionale delle Ricerche, via Abbiatigrasso 207, 27100 Pavia, Italy
and the International Center for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy

We have described previously that, during S-phase, human DNA ligase I is phosphorylated on Ser66, a casein kinase II site. Here we investigate the phosphorylation status of DNA ligase I during the cell cycle by gel shift analysis and electrospray mass spectrometry. We show that three residues (Ser51, Ser76, and Ser91), which are part of cyclin-dependent kinase sites, are phosphorylated in a cell cycle-dependent manner. Phosphorylation of Ser81 occurs at G1/S transition and depends on a cyclin binding site in the C-terminal part of the protein. This modification is required for the ensuing phosphorylation of Ser76 detectable in G2/M extracts. The substitution of serines at positions 51, 66, 76, and 91 with aspartic acid to mimic the phosphorylated enzyme hampers the association of DNA ligase I with the replication foci. We suggest that the phosphorylation of DNA ligase I and possibly other replicative enzymes is part of the mechanism that directs the disassembly of the replication machinery at the completion of S-phase.

In mammalian cells, DNA replication takes place in specific subnuclear compartments, called replication factories, that contain many enzymes and factors involved in this process (1–6). The number, distribution, and size of replication factories vary during S-phase according to a precise program (7–9). In addition, replication factories disassemble in response to chemically induced double strand breaks (10). How these dynamic processes are controlled is still unknown; however, post-translational modifications of replicative factors are likely involved.

DNA ligase I (LigI) is responsible for joining adjacent Okazaki fragments generated during the lagging strand synthesis (11, 12). According to its role in DNA replication, the protein is recruited to replication factories during S-phase (5, 13). The recruitment is directed by a short protein motif termed “replication factory-targeting sequence” (RFTS) (14) that also mediates the interaction with the proliferating cell nuclear antigen (PCNA) (15, 16). A similar motif has been identified in a wide range of proteins involved in DNA replication (17). It is therefore conceivable to consider PCNA as the recruiter that controls the local concentration of many factors required for the proper DNA duplication. The RFTS/PCNA binding site of human LigI is located in the N-terminal region of the protein (residues 1–216) that, although dispensable for the in vitro activity, is essential for the function of the enzyme in vivo (18, 19). In the same region are present several serines that are part of consensus sites for protein kinases and are phosphorylated in vitro (20–22). We have shown previously that human LigI is phosphorylated in a cell cycle-dependent manner on Ser66, which is part of a casein kinase II site (23). The functional role of this modification is still unknown; however, in vitro phosphorylation of human LigI by casein kinase II makes the enzyme no longer responsive to stimulation by PCNA (24).

Additional residues of human LigI undergo in vivo phosphorylation, and, in M-phase the enzyme exists in a hyperphosphorylated form that does not coimmunoprecipitate with PCNA (23). The residues modified in the hyperphosphorylated form of human LigI have not yet been identified.

In this article we report the identification, by electrospray mass spectrometry and gel mobility analysis, of three serines in the N-terminal domain of human LigI that are phosphorylated in a cell cycle-dependent manner. These serines are part of cyclin-dependent kinase (CDK) sites, are in vitro substrates of the cdk2/cyclin A complex, and their in vivo modification depends on a cyclin binding motif (Cy) located in the C-terminal region of the protein. Phosphorylation of one of these serines (Ser23) at the G2/S border is required for the appearance of the hyperphosphorylated form of the enzyme detectable in G2-phase. Substitution of these serines with the phosphoserine-mimetic aspartic acid hampers the recruitment of human LigI to sites of DNA synthesis supporting the idea that phosphorylation can be one of the mechanisms controlling the dynamics of replication factors during S-phase.

EXPERIMENTAL PROCEDURES

Plasmids and Recombinant Proteins—All the human LigI mutants expressed in mammalian cells were C-terminally fused to the muscular actin epitope recognized by the HUC1-1 monoclonal antibody (ICN Pharmaceuticals) and were obtained with suitable primers and PCR-mediated mutagenesis of the pLigI-Tag plasmid described previously (5, 14). Plasmids were verified by DNA sequencing (Thermo Sequenase™ Cy™5.5 Amersham Biosciences). The substitution mutants S51A, S76A, and S91A are human LigI mutants in which serine at positions 51, 76, or 91 were substituted with alanine. S51A/S66A/S76A/S91A (which we call the “4A mutant”) and S51D/S66D/S76D/S91D (which we call the “4D mutant”) are mutants in which serines 51, 66, 76, and 91 were substituted with alanine and aspartic acid respectively. R678A/...
LigI Phosphorylation during the Cell Cycle

R679A/Q808A/L681A (which we refer to as ‘Ala767–786’-mutant) is a mutant in which residues 678–811, corresponding to the C-terminus, were replaced with four alanines. L/F/G/I is a mutant in which phenylalanines at position 8 and 9 are substituted with glycine (14). The deletion mutants are as follows. ΔNLS lacks amino acids 119–131, corresponding to the nuclear localization signal (NLS) of the protein (Δ14). Δ31–118, Δ312–216, Δ378–378, and Δ1–216, lack the corresponding amino acid residues. ΔQRQL lacks amino acids 678–881, corresponding to the C motif. ΔNLSΔQRQL lacks both the NLS and the C motif. Oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany). To express human cyclin A fused to glutathione S-transferase (GST), the cyclin A cDNA was cloned into the EcoRI site of the pGEX-2T plasmid (Amersham Biosciences) in-frame to the C terminus of the GST open reading frame. The recombinant His-LigI and His6-Ala678–786 mutant in 100 µg of recombinant protein (rhLigI) were incubated in 80 µl of kinase buffer (50 mM Tris- HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, and 100 µM ATP) containing 50 mM NaF and 5 mM β-glycerophosphate for 30 min at 30 °C in the presence of 2 µg of the recombinant cdk2/cyclin A active complex. Recombinant cdk2/cyclin A active complex was kindly supplied by Dr. Jerome Breton, Pharmacia Italia S.p.A., Nerviano, Italy. The specific activity (800 nmol/min/ml) was measured with the His-tagged peptide. To assess the role of the Cy motif in LigI phosphorylation, 4 µmol of baculovirus-expressed His6-LigI and His6-Ala767–786 were incubated in 10 µl of kinase buffer containing 10 µCi [γ-32P]ATP, 3000 Ci/mmol for 30 min at 30 °C in the presence of increasing concentrations of cdk2/cyclin A kinase. Reaction products were resolved on 7.5% polyacrylamide SDS gels (SDS-PAGE). Phosphorylation was quantitated by PhosphoImager (Amersham Biosciences). The 23-mer synthetic peptide used as a competitor in kinase assays was purchased from NeoSystem, Strasbourg, France.

Mass Spectrometry—Carboxymethylated proteins were loaded on a 7.5% SDS-PAGE and digested with trypsin or endoproteinase V8. Similar results were also obtained by digesting the immunopurified protein in subphosphate precipitation without loading them on an SDS gel. Trypsin digestion was carried out overnight at 37 °C in 100 µl of ammonium carbonate buffer, pH 8.0, using 10 µl of enzyme (0.1 µg/µl). Digestions with endoproteinase V8 were performed for 2 and 4 h and overnight at room temperature in 100 µl of sodium phosphate buffer, pH 7.3, using 10 µl of enzyme (0.1 µg/µl). The products of digestion were separated by micro high performance liquid chromatography and analyzed by electrospray mass spectrometry (Finnigan LCQ DECA, Thermo-Finnigan Corp., San Jose, CA). Phosphorylated residues were identified by looking at the 80-Da increase in the mass due to the presence of the phosphate group.

Western Blot Analysis—Western blot analysis of total cell extracts was as described previously (10). Primary antibodies were revealed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence systems (Super Signal West Dura Extended, Pierce, for tagged proteins; ECL, Amersham Biosciences, for endogenous LigI and α-tubulin). Rainbow recombinant proteins (Amersham Biosciences) were used as molecular weight markers. To better appreciate the differences between phosphorylated and non-phosphorylated LigI, SDS-PAGE were run until the yellow marker (75 kDa) reached the bottom of the gel. To verify that the wild type protein and the different mutants had the same apparent molecular mass when dephosphorylated, proteins were immunoprecipitated with the anti-tag antibody from extracts of transfected cells and dephosphorylated with alkaline phosphatase as described previously (23).

RESULTS

Cell Cycle-dependent Modification of Human LigI—We have reported previously that phosphorylation of human LigI during cell cycle affects the electrophoretic mobility of the enzyme in SDS-PAGE (23). Indeed, three forms of LigI could be distinguished by Western blot analysis of cells synchronized at different moments of the cell cycle (Fig. 1A). Two bands are detectable in cells stacked at the G1/S boundary with mimosine. Entry in S-phase (release from a double thymidine block) is accompanied by the disappearance of the faster migrating form, suggesting that conversion of one protein band into the other takes place from late G1 to early S-phase. Finally a third protein form, showing a further reduced electrophoretic mobility, is detectable in M-phase extracts obtainable from nocodazole-arrested cells. We took advantage of the fact that the same pattern was detectable with the overexpressed protein (Fig. 1B), and we sought to analyze phosphorylation of LigI by studying the electrophoretic mobility of different mutants expressed in transiently transfected COS7 cells. The day after transfection, cells were synchronized at the mitotic spindle checkpoint by nocodazole and either collected immediately (M) or replated in fresh medium and harvested 6 h later (G1). Total cell extracts were then analyzed in Western blotting with the anti-tag
Fig. 1. Cell cycle-dependent modification of human DNA ligase I. A, total extracts of HeLa cells synchronized at the indicated moments of the cell cycle were analyzed in Western blotting with the anti-LigI rabbit antiserum. B, COS7 cells expressing the epitope-tagged WT LigI were synchronized at different moments of the cell cycle and analyzed by Western blotting with the anti-tag HUC1-1 monoclonal antibody. C, COS7 cells expressing the indicated epitope-tagged proteins were synchronized in the G1- and M-phases. Total cell extracts were analyzed as in panel B. D, COS7 cells expressing the S91A mutant (A91SPPR94) were synchronized at the indicated moments of the cell cycle and analyzed as in panel B. E, COS7 cells expressing the indicated epitope-tagged constructs were synchronized at the G1/S border. Proteins were immunopurified with the anti-tag HUC1-1 antibody and incubated with (+) or without (−) alkaline phosphatase. Reaction products were analyzed in Western blotting with the anti-LigI rabbit antiserum. F, COS7 cells expressing the S76A mutant (A51) were synchronized at the indicated moments of the cell cycle and analyzed as in panel B. G, COS7 cells expressing the indicated epitope-tagged proteins were synchronized in M-phase and analyzed as in panel B. COS7 cells expressing the indicated epitope-tagged proteins (A91, the S51A mutant) were synchronized at the G1/S border (H) or in M-phase (I) and analyzed as in panel B.

antibody. We focused on the N-terminal regulatory domain of the enzyme (residues 1–216) containing several consensus sites for Ser/Thr kinases. The analysis of one of these mutants (∆31–118) showed that the 31–118 region, comprising the already described casein kinase II site (69SEGE69) (23) along with three putative CDK sites (51SPVK54, 76SPAK79, and 91SPPR94), was required for the shift of electrophoretic mobility (Fig. 1C). A different mobility in G1 and M extracts was instead detectable with a complementary mutant (∆132–216) that lacked the portion of the N-terminal domain downstream of the NLS. Both mutants had the same subcellular distribution as the endogenous protein ruling out the possibility that any effect on the electrophoretic mobility could be due to inappropriate localization (23). Further mutants narrowed the region required for the shift to a 41-amino acid stretch (residues 78–118) comprising only one putative CDK site at Ser91 (not shown). Replacement of Ser91 with alanine abrogated the shifts of electrophoretic mobility detectable in early S-phase and mitotic cells (S91A, designated A91 in Fig. 1D). Indeed the S91A mutant migrated as the fastest form of the wild type protein at the G1/S border (Fig. 1E, −). The difference in electrophoretic mobility between the wild type and the mutated LigI was caused by phosphorylation because it was abrogated by phosphatase treatment (Fig. 1E, +). Thus, the behavior of the S91A mutant indicates that phosphorylation of Ser91 occurs at the G1/S transition and is required for the appearance of the slower migrating form of LigI detectable in mitotic cells (see below).

Ser91 Is a Substrate of Cdk2/Cyclin A and Is Phosphorylated in Vivo—The results in the previous section raise the possibility that the cdk2/cyclin A complex, which is required for S-phase progression, could be involved in phosphorylation of Ser91 at the G1/S border. To investigate this aspect, the enzyme expressed in E. coli was challenged in a kinase assay with recombinant cdk2/cyclin A and subjected to electrospray mass spectrometry. The results in Table 1 demonstrate that cdk2/cyclin A can phosphorylate Ser91 along with two additional residues, Ser91 and Ser76, that, as stated before, are part of CDK sites. We have also found that the same three serines are in vitro modified by cdk2/cyclin E (not shown). Although we applied different proteolytic conditions, peptides detected with mass spectrometry covered only 63% of the N-terminal domain, and additional phosphorylated residues could have escaped this analysis.

To verify the phosphorylation status of the three serines in vivo, about 5 μg of endogenous LigI were immunopurified from 4 × 106 mitotic HeLa cells and analyzed by electrospray mass spectrometry as above. Although the detected peptides covered only 49% of the N terminus, this analysis proved that the three serines were indeed phosphorylated in mitotic cells (Table 1). Moreover, these data confirmed the occurrence of Ser96 phosphorylation, which was previously studied with a specific monoclonal antibody (23). Fig. 2 exemplifies the analysis of a phosphopeptide obtained by in vitro phosphorylation of the recombinant protein (panel A) and a phosphopeptide obtained from the endogenous LigI immunopurified from mitotic HeLa cells (panel B). In conclusion, mass spectrometry analysis of the endogenous enzyme demonstrates that Ser91, which is involved in the shift of electrophoretic mobility at G1/S border (see Fig. 1E), is indeed phosphorylated in vivo.

The gel mobility assays in Fig. 1 suggest that modification of Ser91 at the G1/S transition is required for the successive phosphorylation events. To investigate this aspect we determined the phosphorylation pattern of the WT and S91A proteins expressed in transiently transfected COS7 cells. After synchronization in M-phase, the two proteins were immunopurified with the anti-tag antibody and subjected to mass spectrometry. Contrary to the WT protein, the S91A mutant was not phosphorylated on Ser76, suggesting that modification of Ser91 was required for the successive phosphorylation of other residues of LigI. We used the mobility shift assay to follow the phosphorylation of Ser76 during the cell cycle. As shown in Fig. 1F, the S76A mutant underwent the mobility shift between G1/S border and early S-phase but not the successive shift between S- and M-phase. In nocodazole-arrested cells, the S76A mutant migrated faster than the WT protein (Fig. 1G), indicating that phosphorylation of Ser76 causes the slow migrating form of LigI detectable in mitotic cells. Differently from the other two residues, phosphorylation of Ser91 does not perturb the mobility of the protein (Fig. 1, H and I).

Identification of a Cyclin Binding Motif in the C-terminal Domain of Human LigI—We have shown previously that LigI can be co-immunoprecipitated with cyclin A from a nuclear extract of HeLa cells (29). To investigate whether the two proteins could directly interact with each other, we challenged the baculovirus-expressed human LigI in pull-down assays with a GST-cyclin A fusion protein. As shown in Fig. 3, A and B, LigI efficiently bound to GST-cyclin A but not to the sole GST moiety, and the interaction was stable up to 0.3 M NaCl.

Phosphorylation by cdk2/cyclin A usually depends on a Cy motif that represents the docking site for cyclin A on the substrate (30). Interestingly, replacement of a putative Cy site...
(RRQL) at position 678–681 with four alanines affected the *in vitro* phosphorylation of recombinant LigI by cdk2/cyclin A (Fig. 3C). Consistent with this observation, the binding of the Ala*^{678–681}* mutant to GST-cyclin A was reduced (Fig. 3D). However, a residual level of binding and phosphorylation was detectable with the mutated protein suggesting that, at least *in vitro*, additional motifs could mediate the interaction with this kinase. The relevance of the RRQL sequence was confirmed by a second experiment in which increasing concentrations of a peptide containing the putative Cy motif of LigI (residues 668–690) competed the *in vitro* phosphorylation of the recombinant enzyme by cdk2/cyclin A (Fig. 3E).

To verify whether the Cy motif was also important *in vivo*, particularly for Ser*^{91}* and Ser*^{76}* phosphorylation, we expressed in transiently transfected cells the ΔRRQL mutant that was identical to the wild type protein except for the deletion of the Cy motif. After verifying that this deletion did not affect the recruitment to the replication factories (not shown), we studied with Western blotting the mobility of the mutated protein during the cell cycle. Similarly to what we observed with the S91A construct, the electrophoretic mobility of ΔRRQL remained constant throughout the cell cycle (Fig. 3F), indicating that the Cy motif was indeed necessary *in vivo* for phosphorylation of Ser*^{91}* and, hence, of Ser*^{76}*. The results in Fig. 3F, moreover, indicated that the role of the Cy motif in LigI phosphorylation was more important *in vivo* than *in vitro*. We assume that proteins interacting with LigI could contribute to this effect.

The last evidence supporting the involvement of the RRQL motif in protein interactions was derived from a completely different *in vivo* assay. We reported previously that deletion of the nuclear localization signal (residues 119–131, the ΔNLS mutant) did not completely abrogate the nuclear import of LigI in a subset of cells, as if this mutant could enter the cell nucleus piggyback on an interacting protein (5). The analysis of this phenomenon in synchronized cells proved that the nuclear accumulation of ΔNLS mainly took place at G1/S transition, i.e. in parallel with Ser*^{91}* phosphorylation (Table II). A similar phenomenon occurred also with the Δ1–216 mutant that lacked the entire N terminus (not shown), ruling out the involvement of proteins, such as PCNA, that are able to bind the N-terminal regulatory domain of LigI (14). In the hypothesis that the

### Table I

DNA ligase I phosphopeptides identified by electrospray mass spectrometry

The asterisk (*) represents phosphoserine. rhLigI° is recombinant human LigI expressed in *E. coli*, and hLigI is human LigI immunopurified from HeLa cells arrested in M phase.

| Peptide | Position | Peptide sequence | Proteolytic enzyme |
|---------|----------|-----------------|-------------------|
| rhLigI° | 41–54    | EWNGVSESD*S*PVK | Trypsin           |
| rhLigI° | 63–79    | VLGSEGEEDEAL*S*PAK | Trypsin          |
| rhLigI° | 72–86    | DEAL*S*PAKGKPALD | V8 endoproteinase |
| rhLigI° | 87–100   | CSQS*S*PPRATSPE  | V8 endoproteinase |
| hLigI   | 87–112   | CSQS*S*PPRATSPEFENNASLDTSPD | V8 endoproteinase |
| hLigI   | 74–86    | ALS*S*PAKGKPALD  | V8 endoproteinase |
| hLigI   | 51–67    | S*S*PVKRRGKARVLGS*E | V8 endoproteinase |

Fig. 2. Analysis of phosphorylation sites by mass spectrometry. A, recombinant human LigI. B, human LigI immunopurified from HeLa cells. Sections I, base peak chromatogram showing the peptides eluting from a C8 column (1 × 150 mm). Sections II, neutral loss profile detecting a neutral loss of 49 atomic mass units (assuming doubly charged species) due to the release of H3PO4 (98 atomic mass units) from phosphorylated residues. Sections III, Turbo-SEQUEST analysis of the MS² data corresponding to the peptides containing Ser*^{91}* (A) and Ser*^{76}* (B).
incubated with His6-LigI. After washing with 0.15 M NaCl, the bound (LigI (4 pmol) was 7.5% SDS-PAGE and quantitated with a PhosphorImager. subcellular distribution of the reflect an interaction mediated by the Cy motif, we studied the nuclear translocation of /H9004 in which residues RRQL were substituted with four alanines (peptide corresponding to residue 668 phosphorylated in vitro with increasing concentrations of cdk2/cyclin A in the presence of [γ-32P]ATP. Phosphorylated proteins were fractionated on 7.5% SDS-PAGE and quantitated with a PhosphorImager. D, binding of His6-RRQL mutant to cyclin A analyzed as in panel A. E, recombinant LigI (4 pmol) was in vitro phosphorylated with 0.1 pmol of cdk2/cyclin A and [γ-32P]ATP in the presence of the indicated molar excess of a 23-amino acid peptide corresponding to residue 668–690 of human LigI and comprising the Cy motif. The phosphorylated protein was fractionated on 7.5% SDS-PAGE, and the gel was exposed to the PhosphorImager screen. The sequence of the peptide and the Cy motif (underlined) are also shown. F, COS7 cells expressing the tagged ΔRRQL mutant in which residues 678–681 were deleted were synchronized at the indicated moments of the cell cycle. Western blot analysis of total cell extracts with the anti-tag HUC1-1 antibody shows that the protein has the same electrophoretic mobility throughout the cell cycle. G, COS7 cells, expressing the tagged L/F/G mutant unable to interact with PCNA, were synchronized in M-phase and analyzed as in panel F.

TABLE II
Subcellular localization of the ΔNLS mutant in synchronized cells

| Cell cycle phase | C | C/N | N |
|-----------------|---|----|---|
| G₁              | 62 ± 4 | 20 ± 4 | 18 ± 4 |
| G₂/S            | 2 ± 1  | 20 ± 1 | 78 ± 1 |

The fraction of cells in which the ΔNLS mutant was cytoplasmic.

The fraction of cells in which the ΔNLS mutant was distributed in whole cell body.

The fraction of cells in which the ΔNLS mutant was exclusively nuclear.

nuclear translocation of ΔNLS at the onset of S-phase could reflect an interaction mediated by the Cy motif, we studied the subcellular distribution of the ΔNLS/ΔRRQL mutant in which both the NLS and the RRQL motifs were deleted. Contrary to the ΔNLS mutant (Fig. 4, A and B), ΔNLS/ΔRRQL was always confined to the cytoplasm of the transfected cells even during S-phase (Fig. 4, C and D). Thus, a functional Cy motif in the C-terminal catalytic domain of human LigI mediates the interaction with some protein, most likely cyclin A, that enters the nucleus at the G₁/S transition.

Phosphorylation Affects the Recruitment of LigI to Replication Foci—Data in this paper and elsewhere (23) indicate that human LigI is dephosphorylated during the first part of the G₁-phase and is progressively phosphorylated from the G₁/S border to the G₂/M transition. This pattern is suggestive of a role for phosphorylation in controlling the dynamic interaction of the enzyme with other replicative factors and/or with sites of DNA synthesis. In accordance with this hypothesis, we have observed previously that the hyperphosphorylated form of LigI in G₂/M does not co-immunoprecipitate with PCNA (23). To understand whether phosphorylation could affect the localization of LigI, we studied the distribution of mutants in which the four serines phosphorylated in mitotic cells, namely Ser⁶⁶, Ser⁶¹, Ser⁷⁶, and Ser⁹¹ (this study), were substituted with alanine (the 4A mutant) or with the phosphoserine-mimetic aspartic acid (the 4D mutant). The 4A mutant still localized at the replication foci. Accordingly, in a large proportion (68 ± 2%) of transfected cells the 4D mutant did not colocalize with sites of BrdUrd incorporation (Fig. 5A). Western blot analysis confirmed that the WT protein and the two substitution mutants were expressed at comparable levels (Fig. 5B), arguing against the possibility that the different distribution of the 4D mutant could originate from overexpression. We also verified the ability of the 4D mutant to interact with PCNA. In vitro translated WT and 4D proteins were challenged for their interaction with some protein, most likely cyclin A, that enters the nucleus at the G₁/S transition.
DISCUSSION

We have applied different in vitro approaches to study the phosphorylation status of LigI. Phosphorylation of the protein at CDK sites occurs at different moments of the cell cycle and is accompanied by changes of electrophoretic mobility. The under-phosphorylated form of LigI detectable in late G1 is converted to a slower migrating form in early S-phase through phosphorylation of Ser91. This event is a prerequisite for the functional change detectable as a shift of the electrophoretic mobility of the WT protein in mitosis. This phosphorylation probably induces a conformational change detectable as a shift of the electrophoretic mobility of the WT protein. The dual role of phosphorylation in controlling enzymatic activities can also modulate the subnuclear distribution of replicative enzymes. The dual role of phosphorylation in controlling

mental approaches (in vitro versus in vivo) used in these studies. However, we can not rule out that PCNA, acting as a sort of molecular chaperone, could modulate the interaction of LigI with other proteins, among which is cdk2. The importance of the Cy motif is also supported by the observation that at the G1/S border this sequence is sufficient to direct the nuclear accumulation of the ΔNLS mutant (see Table II and Fig. 4). This finding raises the possibility that a complex between ΔNLS and cyclin A is assembled in the cytoplasm at the end of G1-phase and is then imported into the cell nucleus just prior to the onset of DNA replication. There are no indications that such a mechanism could operate during the nuclear import of the endogenous LigI, even though a similar mechanism, i.e. the interaction with cdk2 in the cytoplasm, has been suggested to promote the nuclear import of PCNA, which lacks a canonical NLS.

Our analysis indicates that phosphorylation of Ser91 occurs between late G1 and early S-phase and marks the enzyme until mitosis. This phosphorylation probably induces a conformational change detectable as a shift of the electrophoretic mobility of LigI. Notably, phosphorylation of Ser91 is necessary for the modification of Ser76 detectable in mitotic cells. It is presently unknown how this sequential phosphorylation of CDK sites is achieved and controlled, because in vitro data show that the cdk2/cyclin A complex (as well as cdk2/cyclin E) can phosphorylate all of them. A possible interpretation is that proteins interacting with the N-terminal domain, among which is PCNA, could mask Ser76 during S-phase. An alternative possibility is that a phosphatase associated with LigI could produce a rapid phosphate turnover on Ser76, as was already suggested for Ser76 (23). In this regard, it has been shown recently that DNA polymerase α-prime is associated with a phosphatase as well as with cdk2/cyclin A in human cells (32). Human LigI is also an in vitro substrate for cdc2 kinase (21). Although we do not presently know whether cdc2 contributes to LigI phosphorylation in vivo, the time of phosphorylation of Ser76 is compatible with a role of cdc2/cyclin A complex in this modification. Finally, we can not rule out the possibility that additional residues that escaped our analysis could be phosphorylated by the cdc2/cyclin B complex during mitosis.

A Possible Role of LigI Phosphorylation—Several replicative enzymes are phosphorylated during the cell cycle, but little is known about the function of this modification (33, 34). DNA polymerase α-prime is the best-characterized CDK substrate. This enzyme is sequentially phosphorylated at multiple sites in a cell cycle-dependent manner, and phosphorylation of CDK sites can modulate its ability to initiate SV40 DNA replication in vitro (35, 36). The analysis reported in this paper opens the intriguing possibility that post-translational modifications can also modulate the subnuclear distribution of replicative enzymes.
both a biochemical process and the subnuclear distribution of the proteins involved has already been demonstrated in the case of pre-mRNA splicing. The execution of different steps of the splicing reaction entails successive cycles of phosphorylation and dephosphorylation. Moreover, phosphorylation controls the subnuclear distribution of splicing factors and their association with specific nuclear compartments called speckles (37).

We have mapped at least four serines (Ser\(^{51}\), Ser\(^{66}\), Ser\(^{76}\) and Ser\(^{91}\)) of the N-terminal region of LigI that are phosphorylated in cells chemically arrested in M-phase. Substitution of these serines with alanine or deletion of the Cy motif has no effect on the subnuclear distribution of the protein. In contrast, substitution with aspartic acid to mimic the phosphorylated enzyme drastically hampers the recruitment of LigI to the replication factories. Therefore, the progressive modification of the enzyme drastically hampers the recruitment of LigI to the subnuclear distribution of splicing factors and their contribution has already been demonstrated in the biochemical process and the subnuclear distribution of the proteins involved has already been demonstrated in the case of pre-mRNA splicing. The execution of different steps of the splicing reaction entails successive cycles of phosphorylation and dephosphorylation. Moreover, phosphorylation controls the subnuclear distribution of splicing factors and their association with specific nuclear compartments called speckles (37).

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