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The replication factory targeting sequence/PCNA-binding site is required in G_1 to control the phosphorylation status of DNA ligase I

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The recruitment of DNA ligase I to replication foci in S phase depends on a replication factory targeting sequence that also mediates the interaction with proliferating cell nuclear antigen (PCNA) *in vitro*. By exploiting a monoclonal antibody directed at a phospho-epitope, we demonstrate that Ser66 of DNA ligase I, which is part of a strong CKII consensus site, is phosphorylated in a cell cycle-dependent manner. After dephosphorylation in early G_1, the level of Ser66 phosphorylation is minimal in G_1, increases progressively in S and peaks in G_2/M phase. The analysis of epitope-tagged DNA ligase I mutants demonstrates that dephosphorylation of Ser66 requires both the nuclear localization and the PCNA-binding site of the enzyme. Finally, we show that DNA ligase I and PCNA interact *in vivo* in G_1 and S phase but not in G_2/M. We propose that dephosphorylation of Ser66 is part of a novel control mechanism to establish the pre-replicative form of DNA ligase I.

Keywords: cell cycle/CKII/DNA ligase I/phosphorylation/replication foci

Introduction

DNA ligases play essential roles in DNA metabolism by catalysing the joining of both single- and double-stranded breaks in an ATP-dependent reaction (Kornberg and Baker, 1991). Of the four distinct DNA ligases that have been identified biochemically in mammalian cells (Lindahl and Barnes, 1992; Tomkinson and Levin, 1997) only DNA ligase I (LigI) appears to be involved in DNA replication (Waga *et al.*, 1994). LigI is the main ligase of proliferating cells (Pedrali-Noy *et al.*, 1974; Soderhall, 1976) and its expression is induced by both proliferative (Montecucco *et al.*, 1992) and reparative (Montecucco *et al.*, 1995a) stimuli. The stability of the protein and of its mRNA (Lasko *et al.*, 1990; Montecucco *et al.*, 1992) indicates that control of LigI expression at the transcriptional level takes place over a long time period (proliferating versus resting cells), and suggests that short-term regulation occurs at a post-translational level.

The human DNA ligase I cDNA encodes a 102 kDa polypeptide (hLigI) (Barnes *et al.*, 1990) organized in two well-defined functional domains: a C-terminal catalytic domain (residues 217–919), which shares a significant level of homology with all the ATP-dependent DNA ligases, and a hydrophilic N-terminal region (residues 1–216), which is not necessary for the catalytic activity and that has no counterpart in the other types of DNA ligases so far described in mammalian cells or in yeast. Although dispensable for the *in vitro* DNA-joining activity, the N-terminal domain is essential *in vivo* and its integrity is required for an ectopically expressed enzyme to rescue the lethal phenotype observed in mouse stem cells bearing a knocked out *LigI* gene (Petrini *et al.*, 1995). A clue about the function of the N-terminal domain came from the observation that this region by itself is able to inhibit a cell-free DNA replication assay probably by preventing the interaction of hLigI with the replisome (Mackenney *et al.*, 1997). More recently, it was shown that the N-terminal domain contains all the protein determinants necessary for the nuclear localization of the protein, its recruitment to sites of ongoing DNA replication, the so-called replication factories (Hozak *et al.*, 1993), and interaction with the proliferating cell nuclear antigen (PCNA) (Montecucco *et al.*, 1995b, 1998; Cardoso *et al.*, 1997; Levin *et al.*, 1997). The molecular mechanisms that govern these interactions are still largely unknown but post-translational modifications are most likely to be involved. With regard to this, it is worth underlining that several putative phosphorylation sites have been identified in the N-terminal domain by sequence analysis (Barnes *et al.*, 1990; Savini *et al.*, 1994) and LigI was shown to be the *in vitro* substrate for a number of protein kinases, including CKII and p34 kinase (Prigent *et al.*, 1992). Finally, it was shown that hLigI is phosphorylated *in vivo* at multiple sites (Prigent *et al.*, 1992), but the modified residues and the functional role of these modifications are still to be determined.

In this paper we begin to address the *in vivo* phosphorylation of hLigI and we report the identification and characterization of a phospho-epitope recognized by monoclonal antibody (mAb) 1A4 and located in the N-terminal domain of the enzyme. The modified serine (Ser66) is part of a strong CKII consensus site and is phosphorylated in a cell cycle-dependent manner. We found that the cell cycle-dependent modification of Ser66 is controlled by dephosphorylation in G_1 and probably in S phase. Dephosphorylation in G_1 requires the nuclear localization of the enzyme and a functional PCNA-binding site (Montecucco *et al.*, 1998). Notably hLigI interacts with PCNA *in vivo* both in G_1 and S phase but not in G_2/M.

Results

Isolation of a mAb against a hLigI phospho-epitope

Monoclonal antibodies were raised against the baculovirus-expressed human DNA ligase I (rhLigI). Among

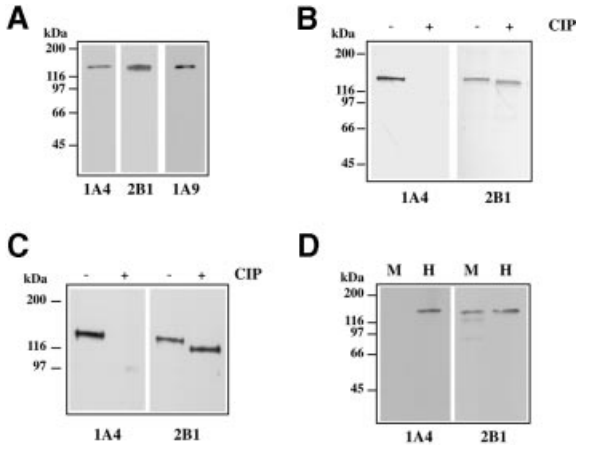


Fig. 1. Characterization of 1A4 and 2B1 mAbs. (**A**) Western blot analysis of HeLa cell extracts with 1A4, 2B1 and 1A9 mAbs. Total cell extract (1 μg) was run on a standard 7.5% SDS–polyacrylamide gel, transferred to a nitrocellulose filter and probed with 1A4 and 2B1 mAbs at a 1:2000 dilution. The anti-DNA ligase I 1A9 mAb (kindly provided by Professor Tomas Lindahl) was used as a control. The antigen–antibody complexes were revealed with a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Pierce) and a chemiluminescent substrate (Super Signal, Pierce). (**B**) 1A4 mAb is specifically directed to a phospho-epitope. Purified rhLigI was incubated with 1 U of CIP (+) or only in phosphatase buffer (–) and analysed by Western blotting with 1A4 and 2B1 mAbs (dilution 1:1000) and alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Promega). (**C**) hLigI was immunoprecipitated from HeLa cell extract as described in Materials and methods, incubated in the presence (+) or in the absence (–) of 1 U of CIP and run on SDS–PAGE (7.5% acrylamide:0.09% bis-acrylamide, to increase the difference in electrophoretic mobility between the phosphorylated and dephosphorylated protein). Proteins were transferred onto a nitrocellulose filter and probed with 1A4 and 2B1 mAbs as described in (A). (**D**) 1A4 mAb does not recognize the murine DNA ligase I. Western blot analysis of human HeLa (H) and murine NIH 3T3 (M) cell extracts with 1A4 and 2B1 mAbs performed as described in (A).

them we selected two independent clones, 2B1 and 1A4, which in HeLa cell extracts recognized a single protein with the same electrophoretic mobility as hLigI, as revealed by the anti-LigI 1A9 mAb (kindly provided by Professor Tomas Lindahl; Figure 1A). The specificity of binding was also confirmed by the fact that recognition of the epitope in Western blotting was entirely competed by an excess of rhLigI (not shown). The two mAbs exhibited a different behaviour in indirect immunofluorescence (Figure 2A). 2B1 mAb stained all HeLa cell nuclei revealing the typical cell cycle-dependent sub-nuclear distribution of LigI, punctated in S phase and homogeneous during the remaining part of the cell cycle (Montecucco *et al.*, 1995b). In contrast, 1A4 mAb decorated only a subset of nuclei, most of which showed the punctated pattern distinctive of S phase. To verify whether sites recognized by 1A4 mAb coincided with replication factories, HeLa cells were synchronized at different moments of S phase, labelled with bromodeoxy uridine (BrdU) for 45 min prior to fixation and immediately stained with anti-BrdU and 1A4 mAbs. Figure 2B shows the distribution of BrdU and 1A4 epitope in cells harvested 1, 5 and 8 h after releasing from the metabolic block. Co-localization of 1A4 epitope with sites of BrdU incorporation was detectable throughout S phase. On the other hand, in a proportion of cells harvested after 8 h, the enzyme was

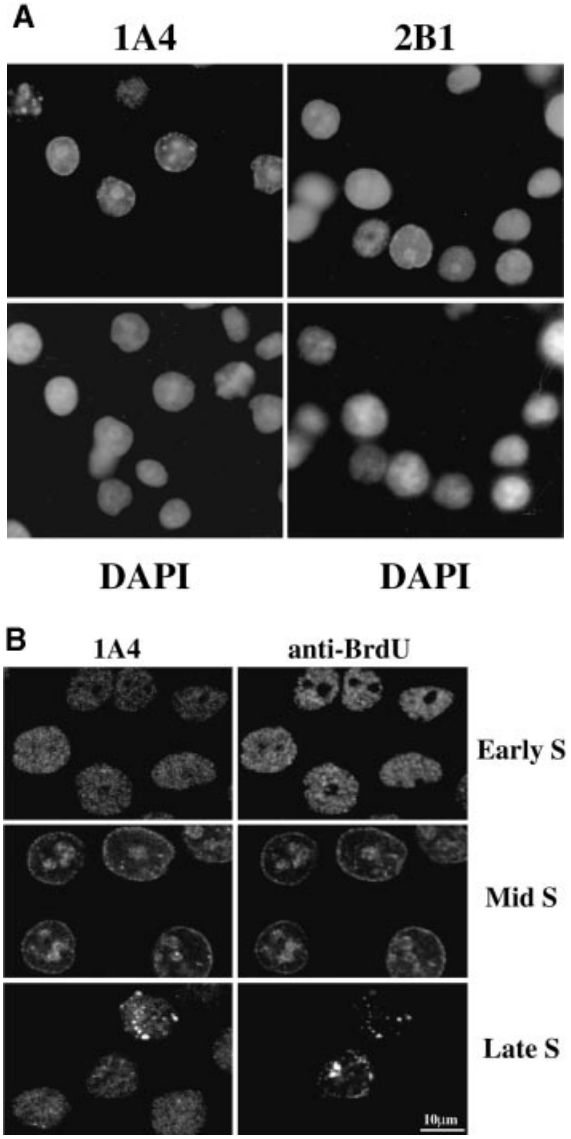
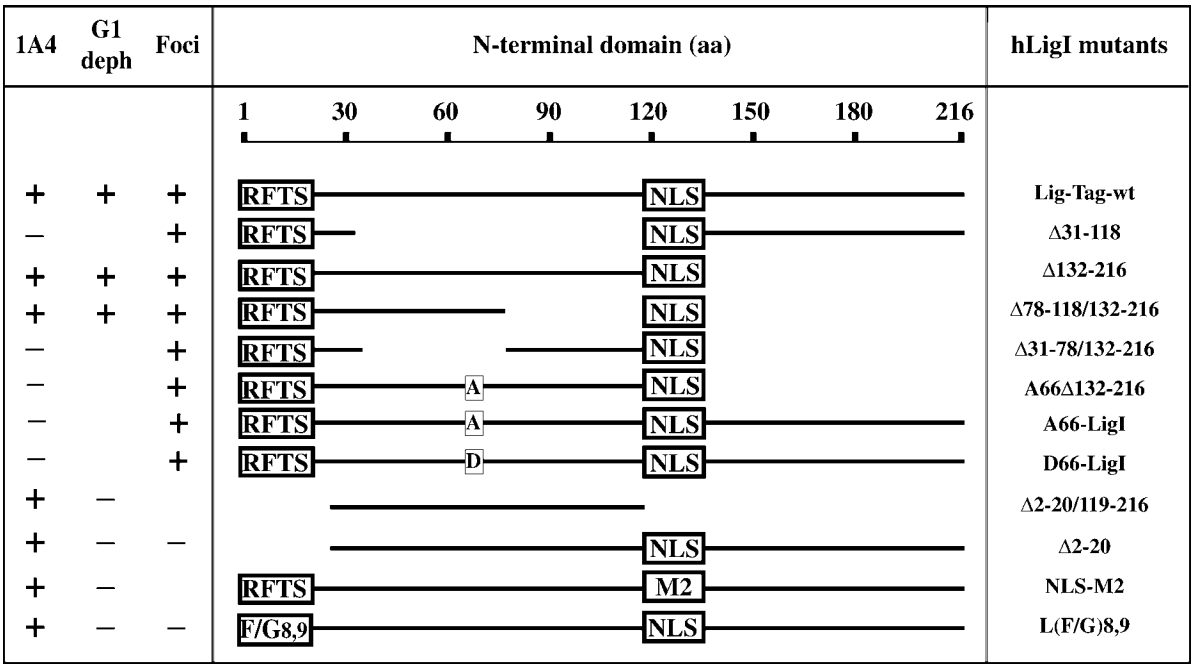


Fig. 2. (**A**) 1A4 mAb detects a subset of HeLa cell nuclei. Exponentially growing HeLa cells were methanol-fixed and immunostained either with 1A4 or 2B1 mAbs and rhodamine-conjugated sheep anti-mouse IgG secondary antibody. Cell nuclei were stained with DAPI. Specimens were examined and photographed with a Leitz Orthoplan microscope. (**B**) HeLa cells were synchronized at the G_1/S border by two successive thymidine blocks. In order to label replication factories, cells were incubated with BrdU for 45 min prior to fixation. Cells were co-stained with 1A4 mAb and FITC-conjugated anti-BrdU mAb. Antigen–antibody complexes were visualized by confocal laser microscopy. Images of the same cells stained with the two antibodies are shown. Early S, 1 h after release from the thymidine block; Mid S, after 5 h; Late S, after 8 h.

no longer exclusively confined to the replication factories but homogeneously distributed throughout the nucleoplasm, presumably as a consequence of the disassembly of the replication factories in late S–early G_2 (Figure 2B). We interpreted the results of the immunofluorescence analysis as an indication that the 1A4 epitope could originate from a cell cycle-dependent post-translational modification of LigI. Since both the antigen and the cellular enzyme have been shown to be phosphorylated (Prigent *et al.*, 1992; Gallina *et al.*, 1995), we asked whether 1A4 mAb was directed toward a phospho-epitope. Thus, 2B1 and 1A4 mAbs were challenged in Western blot

Table I. Schematic representation of the hLigI mutants used in this study



All mutants consist of the entire C-terminal catalytic domain (residues 217–919) preceded by a mutated N-terminal regulatory region (residues 1–216) that contains the indicated deletions and/or substitutions. Mutants were produced with suitable primers and PCR-mediated mutagenesis. All the indicated constructs, including the wild-type protein (Lig-Tag-wt), bear at their C-terminus an epitope recognized by HUC1-1 mAb. The NLS (residues 119–131) and the signal that directs the polypeptides to the replication factories (RFTS, residues 1–20) are indicated. The ability of hLigI mutants to associate with replication factories was scored as '+' (targeting proficient) or '–' (targeting deficient). The presence (+) or the absence (–) of the 1A4 epitope (1A4) in the mutated polypetides is indicated. The ability of the different mutants to undergo (+) or not (–) cell cycle-dependent dephosphorylation of Ser66 is also indicated.

against the purified rhLigI, untreated or dephosphorylated with calf intestinal phosphatase (CIP). As shown in Figure 1B, while the ability of 2B1 to recognize rhLigI was not affected by CIP treatment, 1A4 mAb exclusively detected the non-dephosphorylated enzyme. Consistently, 1A4 mAb also failed to recognize the cellular enzyme immunopurified from a HeLa cell extract and then *in vitro* dephosphorylated with CIP (Figure 1C). We concluded that 1A4 mAb is directed toward a phospho-epitope of hLigI. Notably, this epitope is not conserved in mouse, as indicated by the inability of 1A4 to recognize the murine enzyme in Western blotting (Figure 1D).

The 1A4 phospho-epitope overlaps a CKII site in the N-terminal regulatory domain of hLigI

In order to map the 1A4 phospho-epitope, we developed an *in vivo* assay that entailed Western blot analysis of epitope-tagged hLigI mutants expressed in transiently-transfected cells. We first checked whether the 1A4 epitope was located in the N-terminal regulatory domain of hLigI (residues 1–216), where several consensus sites for Ser/Thr kinases were identified by sequence analysis. We expressed in COS7 cells two epitope-tagged hLigI mutants, the first lacking the region between the replication factory targeting sequence (RFTS) and the nuclear localization sequence (NLS) (Δ31-118; Table I), and the second bearing a deletion of the residues of the N-terminal domain downstream of the NLS (Δ132–216; Table I). Western blot analysis of total cell extracts prepared 48 h after transfection (Figure 3A) showed that 1A4 mAb only recognized the Δ132–216 mutant thus locating the epitope between residues 31 and 118. Further mutants (see Table I) narrowed the interval to a stretch of 47 amino acids (residues 31–77; Figure 3A) comprising five serines and one threonine. We focused on Ser66, which is part of a strong CKII consensus site (S_{66}EGEEEDE; Figure 3). The failure of 1A4 mAb to recognize the A_{66}Δ132–216 mutant in which an alanine was substituted for Ser66 (Figure 3A; Table I) proved that the epitope is produced through phosphorylation of this serine. To test whether the epitope could result from the phosphorylation of hLigI by CKII, the recombinant enzyme was dephosphorylated *in vitro* with CIP, purified on phosphocellulose and re-phosphorylated *in vitro* with recombinant CKII. As shown in Figure 3B, 1A4 mAb recognized only the re-phosphorylated rhLigI. Altogether these results demonstrate that the 1A4 epitope is produced through phosphorylation, most likely by CKII, of Ser66. This finding explains why 1A4 mAb fails to recognize the murine LigI which, even though containing a putative CKII site at the same relative position (S_{65}CEGEDEDE), differs in sequence from the human enzyme.

Phosphorylation of Ser66 is cell cycle-dependent

The indirect immunofluorescence experiment in Figure 2A shows that phosphorylation of Ser66 is cell cycle regulated. To investigate this phenomenon in more detail, HeLa cells were synchronized in mitosis by nocodazole and shake-off treatment, and then released from the block by replating in fresh medium. Total cell extracts were prepared from mitotic cells and from cells harvested 1, 2, 3, 6, 9, 12, 15 and 21 h after removing the drug. The efficacy of

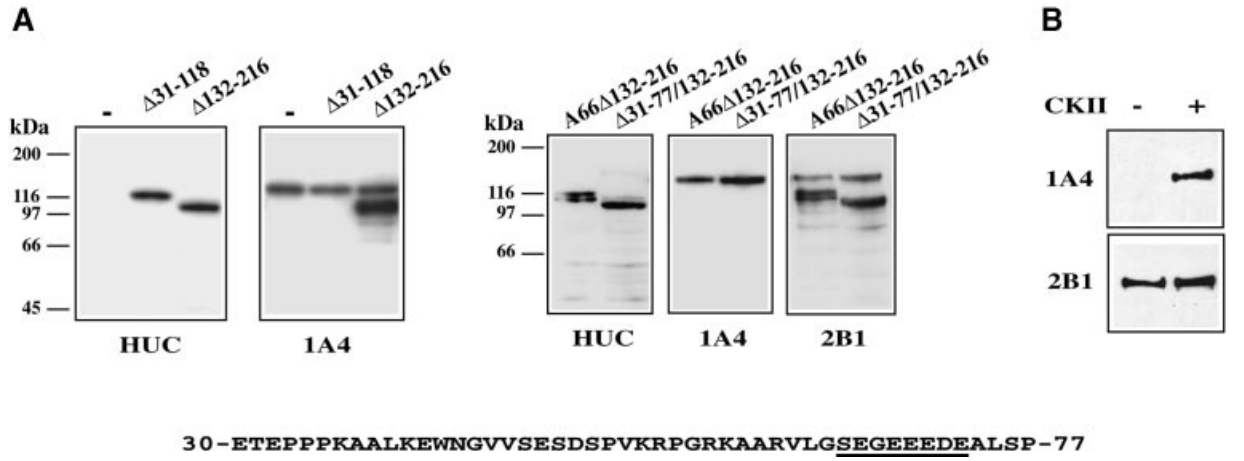


Fig. 3. Identification of the 1A4 phospho-epitope. (**A**) The indicated constructs were transfected into COS7 cells. Total cell extracts were prepared and analysed by Western blotting with anti-tag HUC1-1 (1:1000 dilution), 1A4 and 2B1 mAbs. The Western blot assay was as described in Figure 1A. The position of molecular mass markers is indicated on the left. (**B**) Recombinant rhLigI (10 μg) was dephosphorylated *in vitro* with 10 U of CIP. An aliquot was rephosphorylated with 0.4 mU of recombinant CKII. Both the dephosphorylated and re-phosphorylated proteins were analysed by Western blotting with 1A4 and 2B1 mAbs under conditions described in Figure 1A. At the bottom of the figure is the sequence of the hLigI region spanning residues 30–77. The CKII consensus site is underlined.

synchronization was assessed both by FACS (not shown) and by Western blot analysis of total cell extracts with anti-cyclin A, anti-cyclin B and anti-cyclin E antibodies (Figure 4A). It is known that cyclin A is degraded in mitosis (normally before the nocodazole block), is absent in G_1 and starts to accumulate as cells reach the G_1/S border. Cyclin B is degraded as soon as nocodazole is washed out and cells are allowed to complete mitosis. Finally cyclin E is detectable only in a narrow window centred around the G_1/S border (Pines and Hunter, 1994; Sherr, 1994; Clute and Pines, 1999). As shown in Figure 4A, cyclin A was barely detectable in extracts prepared either from mitotic cells or from cells grown for up to 12 h in nocodazole-free medium, in agreement with the results obtained by Pagano *et al.* (1992). In contrast, the cyclin B level was high in mitosis and during the first hour of incubation in the absence of the drug, and fell drastically during the successive hour. Cyclin E was detectable only in extracts prepared 9, 12, 15 and 21 h after reseeding cells in fresh medium and peaked between 12 and 15 h. The same cell extracts analysed with anti-cyclin antibodies were then probed with 1A4 and 2B1 mAbs. As expected, comparable levels of 2B1 epitope, and hence of hLigI, were detectable throughout the experiment. In contrast, the level of 1A4 epitope changed during the cell cycle (Figure 4A): it was maximal in mitosis, decreased during the first 2 h of growth in fresh medium and was undetectable for most of G_1 (3, 6 and 9 h). The reappearance of the epitope 12 h after release from the block occurred concomitantly with the increase in cyclin A levels. Notably, mitotic levels of 1A4 epitope were not reached even in late S/G_2 phase (21 h after replating in nocodazole-free medium). In conclusion, phosphorylation of Ser66 starts as soon as cells enter S phase, although the level of 1A4 epitope increases mostly after completion of S phase. Western blot analysis of extracts prepared from cells in G_1 (6 h), S phase (12 and 15 h) or mitosis (M) and fractionated in SDS–PAGE, under conditions that amplify differences in electrophoretic mobility, showed that the apparent molecular mass of hLigI was significantly higher in M than during the rest of the cell cycle (Figure 4B). This difference in electrophoretic mobility is likely to be due to a change in the phosphorylation level since, as we showed in Figure 1C, the apparent molecular mass of hLigI is significantly affected by this post-translational modification. It is worth underlining that the fraction of the enzyme stained by 1A4 in S phase still migrated faster than mitotic hLigI, suggesting that the change in electrophoretic mobility is due to phosphorylation of as yet unidentified residues. This interpretation is in agreement with the results of Prigent *et al.* (1992), according to which the enzyme is a substrate for $p34^{cdc2}$ *in vitro*.

In summary, the results in this section seem to indicate that the cell cycle-dependent phosphorylation of Ser66 can distinguish the post-replicative form of hLigI in S and G_2 from the pre-replicative enzyme in G_1. This issue will be addressed further in the Discussion.

Dephosphorylation of Ser66 requires the RFTS

The proximity of Ser66 to both the RFTS (residues 1–20; Montecucco *et al.*, 1998) and the NLS signals (residues 119–131; Montecucco *et al.*, 1995b) prompted us to investigate whether phosphorylation of this residue could affect the nuclear and/or sub-nuclear targeting of hLigI. Thus, we produced two tagged mutants of hLigI in which Ser66 was replaced either with an alanine or with an aspartic acid residue to mimic the permanently dephosphorylated or permanently phosphorylated enzyme, respectively. Mutants were expressed in transiently-transfected cells and their localization was assessed by indirect immunofluorescence with anti-tag HUC1-1 mAb. As shown in Figure 5A, both mutants were nuclear and efficiently recruited to replication factories in S phase, ruling out the possibility that phosphorylation of Ser66 alone could affect the sub-cellular distribution of LigI during the cell cycle.

Next we explored the alternative possibility that the sub-cellular distribution of the enzyme could instead influence the phosphorylation status of Ser66. In order to do this, we tested the ability of 1A4 mAb to recognize the Δ2-20/119–216 mutant (Table I), which lacks both the

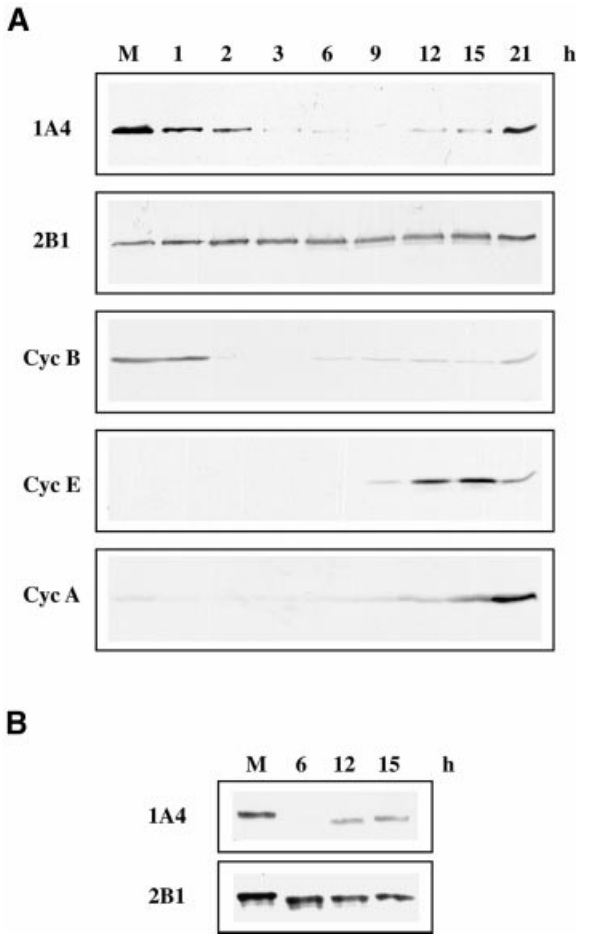


Fig. 4. Cell cycle-dependent phosphorylation of Ser66. HeLa cells were synchronized in mitosis by a 16 h nocodazole (40 ng/ml) and shake-off treatment. (**A**) Mitotic cells were divided into nine aliquots. One aliquot (M) was immediately lysed in sample buffer to be successively analysed by Western blot. The remaining aliquots were reseeded in fresh medium and harvested 1, 2, 3, 6, 9, 12, 15 and 21 h later. Equal numbers of cells were analysed by Western blotting with 1A4 and 2B1 mAbs, anti-cyclin A rabbit antiserum, anti-cyclin B and anti-cyclin E mAbs after fractionation on a 10% SDS–PAGE. (**B**) Samples were run on 7.5% acrylamide:0.09% bis-acrylamide SDS–PAGE to increase the difference in electrophoretic mobility among proteins with different levels of phosphorylation.

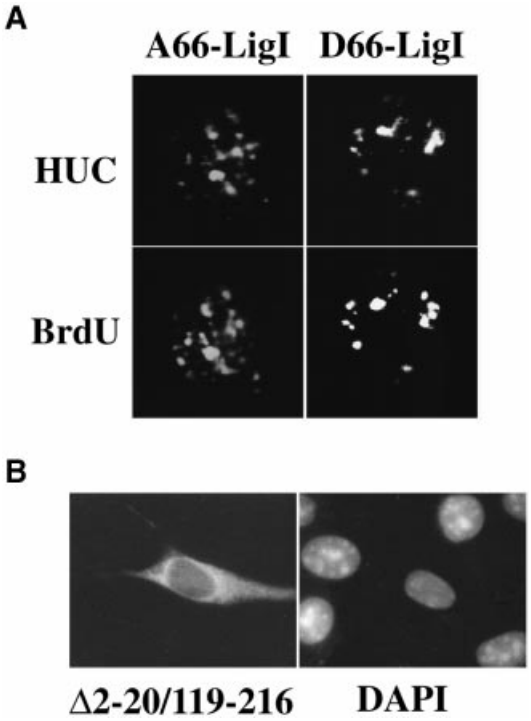


Fig. 5. (**A**) Recruitment of Ser66 substitution mutants to the replication factories. A66-LigI and D66-LigI were transfected into 46BR.1G1 cells and their sub-cellular distribution was determined by indirect immunofluorescence 48 h later. For 45 min prior to fixation, cells were grown in BrdU-containing medium in order to label replication factories. Cells were co-stained with HUC1.1 mAb and a FITC-conjugated anti-BrdU mAb as described in Montecucco *et al.* (1995). Confocal laser images of the transfected cells are shown. (**B**) Cytoplasmic phosphorylation of Ser66. The Δ2-20/119–216 mutant, which is confined to the cell cytoplasm, was expressed in NIH 3T3 cells and the presence of the 1A4 phospho-epitope was verified by indirect immunofluorescence. Nuclei were stained with DAPI. Specimens were photographed with a Leitz Orthoplan microscope.

NLS and the RFTS and is therefore confined to the cytoplasm of the transfected cells. Mouse NIH 3T3 cells were selected for this experiment since the murine enzyme is not recognized by 1A4 mAb (see Figure 1D). As shown in Figure 5B, 1A4 stained the cytoplasm of the transfected cells, unequivocally proving that phosphorylation of Ser66 did not require the proper sub-cellular localization of the enzyme.

Finally, we investigated whether the sub-cellular distribution could control Ser66 phosphorylation during the cell cycle. The cytoplasmic Δ2-20/119–216 mutant described above was expressed in COS7 cells, and total cell extracts were prepared either from cells synchronized in mitosis with nocodazole or harvested in G_1, 6 h after releasing from the block. Western blot analysis with both 1A4 and anti-tag HUC1-1 mAbs showed that, contrary to the cellular enzyme, this mutant was still phosphorylated in G_1 (Figure 6A). To rule out the possibility that this difference could be simply due to the overexpression of the recombinant protein (Figure 6A, compare the intensity of the 1A4 signals due to the endogenous and to the transfected protein, respectively), a wild-type epitope-tagged hLigI was challenged in the same assay. The fact that the transfected wild-type enzyme was correctly dephosphorylated in G_1 indicated that the sub-cellular distribution did indeed have a role in the cell cycle-dependent modification of Ser66 (Figure 6B). Surprisingly, however, dephosphorylation rather than phosphorylation appeared to be affected by the sub-cellular localization of the transfected protein. To investigate this aspect further, we tested additional hLigI mutants in the same assay (see Table I). The first mutant (NLS-M2) was confined to the cytoplasm because of amino acid substitutions that inactivate the NLS (Montecucco *et al.*, 1995b). The second mutant, which bore an 84-amino-acid deletion in the N-terminal domain downstream of the NLS (Δ132–216), was nuclear and was recruited to the factories during S phase (Montecucco *et al.*, 1998). The last mutant (Δ2-20) lacked a RFTS and, consequently, although nuclear, was not targeted to the BrdU foci in S phase (Montecucco *et al.*, 1998). As shown in Figure 6C–E, only Δ132–216 was correctly dephosphorylated in G_1, while NLS-M2 remained phosphorylated in G_1 in agreement with the result obtained with the Δ2-20/119–216 mutant

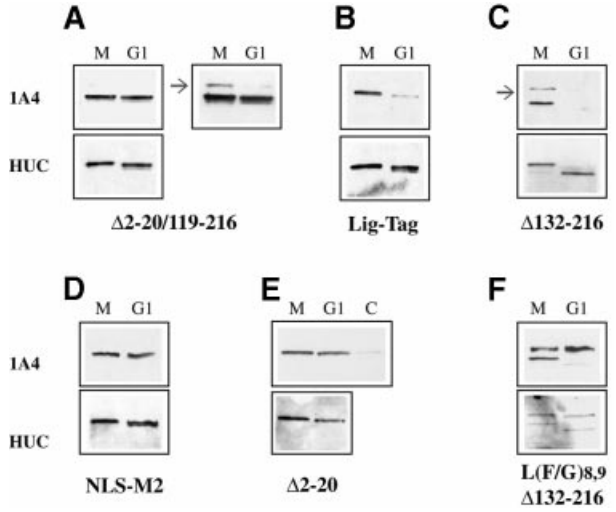


Fig. 6. Identification of the protein motifs required for dephosphorylation of Ser66 in G_1. COS7 cells were transfected with the indicated constructs. The day after transfection, cells were synchronized in mitosis by nocodazole and shake-off treatment. Half the population was immediately lysed in sample buffer (M). The remaining cells were replated in fresh medium and harvested 6 h later (G_1). Total cell extracts were analysed with 1A4 and HUC1-1 mAbs as described in Figure 1A. (**A**) Analysis of the Δ2-20/119–216 mutant. Left and right panels are different exposures of the same filter. The arrow points to the endogenous DNA ligase I. (**B**), (**C**), (**D**) and (**E**) show the behaviour of the wild-type protein (Lig-Tag) and of the indicated mutants (Δ132–216, NLS-M2, Δ2-20). The arrow in (C) points to the endogenous DNA ligase I. Lane C in (E) shows, for comparison, the levels of 1A4 epitope due to the endogenous enzyme in an equivalent amount of non-transfected mitotic cells. (**F**) The behaviour of the L(F/G)8,9 and Δ132–216 mutants co-transfected into COS7 cells.

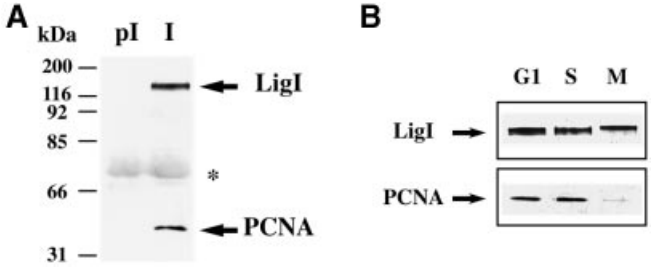


Fig. 7. Co-immunoprecipitation of hLigI and PCNA from HeLa cell extract. (**A**) hLigI was immunoprecipitated from HeLa cell extract as described in Materials and methods. hLigI and PCNA were revealed by Western blotting with 2B1 and PC10 mAbs, respectively. pI, pre-immune serum; I, anti-ligase I rabbit antiserum. The cross-hybridization with rabbit IgG is indicated by an asterisk. (**B**) hLigI was immunoprecipitated from extracts of HeLa cells synchronized in G_1, S phase and mitosis (M); hLigI and PCNA were revealed by Western blotting as above.

(Figure 6A). Unexpectedly, the Δ2-20 mutant which lacked the RFTS did not undergo dephosphorylation in G_1. Since Ser66 is close to the region deleted, the possibility existed that a structural rearrangement occurring in this mutant could affect the recognition of the phosphoresidue. To rule out this possibility, and to understand whether dephosphorylation did indeed require a functional RFTS, we exploited the $L(F/G)_{8,9}$ mutant in which two consecutive phenylalanines in the RFTS were replaced with glycines. We showed previously that this mutant is unable to interact with PCNA and, even though correctly targeted to the cell nucleus, is never recruited to the replication factories (Montecucco *et al.*, 1998). The $L(F/G)_{8,9}$ mutant was co-transfected with the Δ132–216 construct into COS7 cells, and phosphorylation of Ser66 was assessed by Western blotting. As shown in Figure 6F, contrary to the result for Δ132–216, $L(F/G)_{8,9}$ remained phosphorylated in G_1. Thus, Ser66 dephosphorylation in G_1 depends both on the nuclear localization of the enzyme and on the functionality of the RFTS motif, previously thought to be required only during S phase.

In vivo interaction of hLigI with PCNA is cell cycle-dependent

The results in the previous section demonstrate that dephosphorylation of Ser66 in G_1 is prevented by the same mutations that inhibit the interaction of hLigI with PCNA *in vitro* as well as inhibiting the recruitment of the enzyme to the replication factories. It is conceivable, therefore, that both dephosphorylation of Ser66 in G_1 and recruitment to the factories in S phase are correlated to the interaction of hLigI with PCNA. On the basis of this consideration, we decided to test whether such an interaction did occur *in vivo*. We used a rabbit polyclonal antiserum to hLigI to immunoprecipitate the enzyme from a total cell extract prepared from exponentially growing HeLa cells. We then checked by Western blotting whether PCNA could be co-immunoprecipitated under conditions that preserve protein–protein interactions. As a control, the same experiment was performed with the pre-immune serum. As shown in Figure 7A, the polyclonal antiserum, but not pre-immune serum, efficiently co-immunoprecipitated PCNA, which was in fact recognized by the commercial PC10 mAb. Thus, this experiment supported the conclusion, so far based only on *in vitro* data, that there is an interaction between these two replicative proteins. Next we investigated whether the interaction was cell cycle-dependent. Total cell extracts were prepared from cells synchronized in mitosis by nocodazole (Figure 7B, M) or harvested 6 h after removing the drug (G_1). Extracts were also prepared from cells synchronized in S phase by a double thymidine block and then released for 3 h before harvesting (S). As shown in Figure 7B, PCNA was efficiently co-immunoprecipitated from extracts prepared from cells in G_1 and S phase but not in mitosis. Since the interaction with PCNA is detectable only when most of Ser66 is dephosphorylated, it is possible to hypothesize an involvement of PCNA in promoting and/or maintaining the dephosphorylation of Ser66.

Discussion

In vivo phosphorylation of Ser66 of DNA ligase I

In this paper we report the identification and characterization of a phospho-epitope of the N-terminal regulatory domain of hLigI. The epitope is recognized by the 1A4 mAb and is produced *in vivo* by phosphorylation of Ser66, which is part of a CKII consensus site and is in fact modified by this kinase *in vitro*. It was already shown by Prigent *et al.* (1992) that hLigI is a good CKII substrate *in vitro*. In particular, the N-terminal region (residues 1–216) comprises seven S--E CKII consensus sites, two of which have the properties of 'strong' CKII phosphorylation sites: S_{66}EGEEEDE and S_{141}EDED. The results described here demonstrate that phosphorylation of at least one of

these sites does occur *in vivo* and produces the 1A4 phospho-epitope.

The physiological role of CKII is largely unknown but its importance is suggested by the evolutionary conservation of the enzyme and by the fact that disruption of both *Saccharomyces cerevisiae* genes encoding CKII catalytic subunits is a lethal event (Padmanabha *et al.*, 1990). According to some authors, CKII acts as a priming kinase that, by phosphorylating a protein, produces a substrate for further kinases. On the other hand, CKII has been suggested to modulate directly the function of its target proteins (for reviews, see Pinna and Meggio, 1997; Glover, 1998), which include several transcription factors such as TFIIIB (Ghavidel and Schultz, 1997), ATF1 (Yamaguchi *et al.*, 1998) and NF-κB (Bird *et al.*, 1997). A few replicative enzymes besides DNA ligase I, such as DNA topoisomerase II and DNA polymerase α, have been shown to undergo modification by CKII *in vitro* (Podust *et al.*, 1990; Cardenas *et al.*, 1992). DNA topoisomerase II of both *Drosophila* and mouse cells is phosphorylated *in vivo* at CKII sites and this modification has been suggested to trigger the activation of the enzyme (Ackerman *et al.*, 1988; Saijo *et al.*, 1990). As far as hLigI is concerned, the effect of phosphorylation by CKII on the catalytic activity is still disputed (Prigent *et al.*, 1992; Teraoka *et al.*, 1993).

Our observation that 1A4 epitope co-localizes with sites of DNA replication might suggest a role of Ser66 phosphorylation in controlling the sub-cellular distribution of hLigI. The analysis of a few mutants seems to argue against this possibility. In fact, replacement of Ser66 with an alanine or an aspartic acid residue does not perturb either the nuclear localization or the association of the enzyme with replication factories. We cannot presently rule out that other residues, also subjected to post-translational modifications, might assist Ser66 in controlling the sub-cellular distribution of hLigI. With regard to this, it is worth underlining that the N-terminal regulatory region contains several putative CKII and cdk phosphorylation sites, some of which are likely to be phosphorylated *in vivo* mainly in G_2/M phase.

Phosphorylation could influence the affinity of hLigI for chromatin, as suggested by the experiment shown in Figure 8. Chromatin with a protein:DNA mass ratio of 1:1 was purified through two successive CsCl density gradients from formaldehyde-fixed cells as described by Gohring and Fackelmayer (1997). Western blot analysis of the purified material with 2B1 mAb indicated that at least a fraction of hLigI was cross-linked to DNA under these conditions. Notably the same chromatin preparation was not recognized by 1A4 and therefore the enzyme was not phosphorylated at Ser66. It is known that LigI displays a low affinity for nuclear structures and, in fact, elutes from the nuclei during cell fractionation experiments (Spadari *et al.*, 1971). It is possible, therefore, that the chromatin-bound enzyme corresponds to a fraction of hLigI more tightly in contact with DNA, probably because it is engaged in DNA replication and/or repair. We think that this result is compatible with the idea, discussed below, that phosphorylation of Ser66, and probably of other residues, could modulate the interaction of hLigI with the replisome.

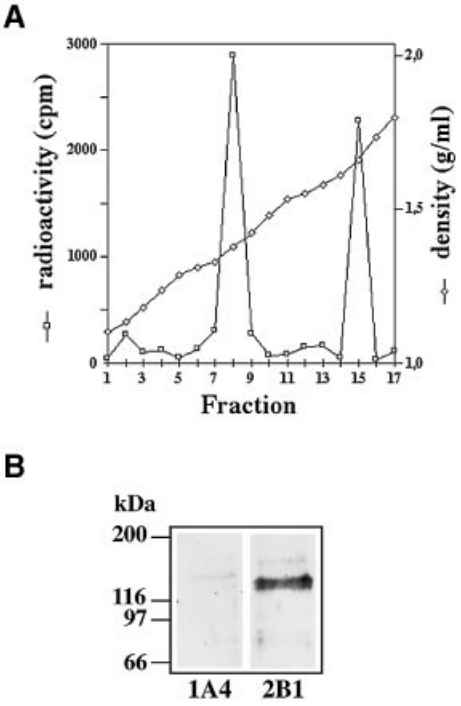


Fig. 8. Absence of the 14A phospho-epitope in the chromatin-bound preparation of hLigI. (**A**) Complexes with a density of 1.42 g/ml, corresponding to a mass ratio of 1:1 (protein:DNA) characteristic of native chromatin, were purified from exponentially growing HeLa cells fixed for 4 min at 37°C with 1% formaldehyde. Purification of DNA–protein complexes by equilibrium centrifugation was performed in two consecutive caesium chloride gradients. To determine the distribution of DNA, cells were labeled for 24 h with [^{3}H]thymidine before cross-linking. Lysed nuclei were centrifuged on a pre-formed gradient and individual fractions were tested for DNA by liquid scintillation counting. Fractions 9–11 were pooled, recentrifuged on an isopycnic caesium chloride gradient, fractionated and analysed as above (not shown). The fraction with a density of 1.4 g/ml was then subjected to Western blot analysis. (**B**) Complexes purified from 3×10^7 cells were TCA-precipitated and resuspended in 100 µl of sample buffer for SDS–PAGE. A fraction corresponding to one-tenth was boiled for 10 min to revert cross-linking, fractionated on a 7.5% SDS–PAGE and analysed by Western blotting with either 1A4 or 2B1 mAbs.

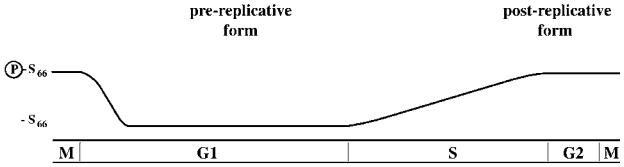


Fig. 9. Schematic representation of the phosphorylation status of Ser66 during the cell cycle. The curve drawn above the diagram of the cell cycle represents the level of 1A4 epitope as determined by Western blot analysis. A high level of 1A4 epitope, and hence of phosphorylated Ser66, is detectable in G_2 and mitosis (post-replicative form of DNA ligase I). The 1A4 epitope is undetectable for most of G_1, indicating that the dephosphorylated Ser66 is a distinguishing feature of the pre-replicative enzyme. Dephosphorylation occurs rather rapidly in early G_1. In contrast, phosphorylation of Ser66 occurs throughout the S phase as indicated by the slow increase in the 1A4 epitope level.

Phosphorylation of Ser66 is controlled during the cell cycle

Contrary to most of the protein kinases described so far, CKII does not seem to be controlled by external stimuli, by cell differentiation or during the cell cycle (Allende and Allende, 1995; Pinna and Meggio, 1997). Thus, CKII

probably acts by providing a kind of 'constitutive' labelling of the modified proteins (Allende and Allende, 1995). It is surprising, therefore, that phosphorylation of Ser66 is regulated during the cell cycle. As shown schematically in Figure 9A, Ser66 is dephosphorylated early in G_1 and remains unphosphorylated throughout this phase. The level of phosphorylated Ser66 gradually increases in S phase and plateaus in G_2/M. Therefore, phosphorylation of Ser66, and probably other residues, could mark hLigI molecules used during a DNA replication event while dephosphorylation would set up a pool of enzyme for the ensuing S phase. Usually a distinction between pre- and post-replicative pools of proteins involved in DNA replication is achieved by cyclin–cdk-mediated phosphorylation. This is, for instance, the case for the p180 and p70 subunits of mammalian DNA polymerase α/primase (Nasheuer *et al.*, 1991) and for mammalian CDC6 (Petersen *et al.*, 1999). It is known that the activity of the cyclin–cdk pairs, and therefore the phosphorylation level of their substrates, is modulated during the cell cycle. Our results suggest the existence of an alternative mechanism to control the phosphorylation status of replicative enzymes. This would achieve a cell cycle-dependent phosphorylation of specific residues, such as Ser66, by means of a constitutive kinase whose activity is counteracted by a cell cycle-dependent dephosphorylation.

Regulation of the phosphorylation status of Ser66 by the RFTS/PCNA-binding site

Our conclusion that phosphorylation of Ser66 is a type of default condition while dephosphorylation is controlled, relies on the behaviour of a few hLigI mutants expressed in transiently-transfected cells. A hLigI mutant (NLS-M2), confined to the cell cytoplasm because of the inactivation of the NLS, is permanently phosphorylated on Ser66, indicating that this mutant is still recognizable by the kinase but not by the phosphatase in the cytoplasm. Unexpectedly, dephosphorylation also requires a functional RFTS, and deletion or inactivation of this motif [Δ2-20 and $L(F/G)_{8,9}$ mutants] results in the constitutive phosphorylation of Ser66 even if the protein is nuclear in both G_1 and S phase. We have reported previously that the RFTS is necessary both for the recruitment of hLigI to replication factories and for the interaction with PCNA *in vitro* (Montecucco *et al.*, 1998). We have shown here that hLigI binds PCNA *in vivo* both in S phase, as expected, and in G_1 but not in mitosis. The observation that the same residue substitutions which abrogate PCNA binding also prevent the dephosphorylated status of Ser66 in G_1, suggests a role for PCNA in this nuclear process. PCNA could bind dephosphorylated hLigI and cover Ser66, thus preventing its phosphorylation. Alternatively, upon binding to DNA ligase I, PCNA could act as a landing pad for a phosphatase or for a kinase inhibitor, and direct hLigI dephosphorylation in G_1. Our data suggest that dephosphorylation of Ser66 could also occur in S phase. This would account for the fact that, although the 1A4 epitope is clearly detectable in the replication factories, its level in S phase increases only slightly (Figure 4). We propose that this behaviour could be correlated to the organization of the mammalian genome into thousands of replicative units or replicons. After a replicon has been completely duplicated, a rearrangement of the replisome is likely to occur and this allows the recycling of the replicative enzymes to another replication unit. It is possible that this rearrangement is accompanied by a cycle of Ser66 phosphorylation/dephosphorylation. At the end of S phase, the inactivation of the dephosphorylation mechanism would produce the increase in the 1A4 epitope observed in G_2/M and would lead to the irreversible inactivation of the replication apparatus as exemplified by the lack of interaction between PCNA and hLigI. In conclusion, our data suggest that the RFTS/PCNA-binding site of hLigI is necessary in G_1 for the interaction with PCNA, which in turn is required for promoting and/or maintaining the dephosphorylated status of Ser66. The hLigI–PCNA complex is then recruited to the replication factories. Phosphorylation of Ser66 by itself is not sufficient to abrogate the interaction with PCNA. On the other hand, phosphorylation of Ser66 and binding to PCNA seem to occur at different times in the cell cycle. In this scenario, the staining of the replication foci by 1A4 is likely to represent a transient disruption of protein interactions within the replisome necessary for the dynamic rearrangements of the replication factories. We speculate that other replication proteins bearing the RFTS/PCNA-binding motif, such as the large subunit of RF-C (Montecucco *et al.*, 1998), MCMT (Chuang *et al.*, 1997) and possibly FEN1 (Li *et al.*, 1995), could undergo a similar phenomenon, thus revealing a novel role for PCNA in cell cycle progression.

Materials and methods

Cells, cell treatments, transfection and immunofluorescence

The SV40 transformed human fibroblast cell line, 46BR.1G1 (ECACC, UK), was grown in complete DMEM medium (Sigma-Aldrich) with 15% FCS. HeLa S3, COS7 and NIH 3T3 cells were grown in complete DMEM medium with 10% FCS as described by Montecucco *et al.* (1992). Synchronization of HeLa S3 cells at the G_1/S border was obtained by growing cells in 2 mM thymidine as described by Stein and Stein (1989). Synchronization in mitosis was obtained by growing HeLa S3 or COS7 cells for 16 h in complete medium containing 40 ng/ml nocodazole. Mitotic cells were then shaken-off, centrifuged and reseeded in fresh medium. Transfections of NIH 3T3, COS7 and 46BR.1G1 fibroblasts, cell fixation and indirect immunofluorescence microscopy were performed as described previously (Weighardt *et al.*, 1995). Epitope-tagged proteins were detected with the anti-muscular actin HUC1-1 mAb (ICN, USA) and a rhodamine-conjugated sheep anti-mouse IgG $F(ab')_2$ fragment secondary antibody (Boehringer Mannheim). To simultaneously detect epitope-tagged polypeptides and sites of DNA synthesis, cells were grown in 50 μM BrdU (Sigma-Aldrich) for 45 min immediately prior to methanol fixation and treated as described by Montecucco *et al.* (1995b). Nuclei were stained with 0.2 μg/ml DAPI (Sigma-Aldrich). Optical sections were obtained using the Bio-Rad MRC-1024 confocal microscope. Micrographs were taken using a Focus Imagecorder Plus (Focus Graphics Inc.) on Kodak Tmax 100 film.

Antibodies

To obtain anti-hLigI mAbs, 8-week-old female Balb/c mice were injected i.p. with purified recombinant hLigI overexpressed in a baculovirus/insect cell system (Gallina *et al.*, 1995). The first i.p. injection consisted of 50 μg of purified protein in 100 μl of sterile PBS/complete Freund's adjuvant (1:1, v/v). Two boosting injections, containing the same amount of antigen but no adjuvant, were administered at 2-week intervals. Three days after the last injection, the splenocytes were fused with myeloma line Sp2/0AG14 and cultured in modified RPMI 1640 supplemented with human endothelial culture supernatant as described previously (Negri *et al.*, 1992). The hybridoma supernatants were screened by ELISA using the purified antigen. Positive clones were expanded in massive culture and tested in Western blotting against total cell extracts to select for clones that recognize a single band corresponding to hLigI.

Anti-DNA ligase I 1A9 mAb was kindly provided by Professor Tomas Lindahl (ICRF, Clare Hall Laboratory, South Mimms, UK). Rabbit antiserum (Lβ) to baculovirus-expressed rhLigI was purchased from Primm (Milano, Italy). Rabbit antiserum to cyclin A was kindly provided by Dr Giulio Draetta (IEO, Milano, Italy). Anti-cyclin B (GNS1) mAb was purchased from Santa Cruz Biotechnology. Anti-cyclin E was purchased from Oncogene.

Immunoprecipitation and co-immunoprecipitation

A total of 2×10^6 cells were lysed for 10 min in 0.5 ml of buffer A (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100) or buffer B (10 mM Tris–HCl pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$) containing 0.2 mM AEBSF (Inalco), 1 μg/ml pepstatin A (Sigma-Aldrich) and 1 μg/ml leupeptin (Sigma-Aldrich) for immuno- or co-immunoprecipitation experiments. The lysate was sonicated twice at 50 W for 20 s and centrifuged at 12 000 *g* for 10 min to remove cell debris. The supernatant was then incubated for 1 h at 4°C with 10% suspension of protein A–Sepharose beads (Pharmacia Biotech) coupled with 4 μl of a rabbit anti-rhLigI antiserum (Lβ). After centrifugation the pellet was washed 2× with buffer A and 2× with buffer A, adjusted to 0.5 M NaCl for immunoprecipitation and 4× with buffer B for co-immunoprecipitation of PCNA. The immunoprecipitated material was then analysed in Western blotting with mAbs to hLigI (1A4 and 2B1) and to PCNA (PC10, Santa Cruz). An anti-mouse IgG minX (Jackson Immuno Research Laboratories) was used as secondary antibody.

Purification and in vitro phosphorylation of rhLigI

rhLigI (10 μg) (Gallina *et al.*, 1995) was dephosphorylated in a final volume of 200 μl of 1× NEB buffer (Biolabs) with 10 U of CIP (Biolabs) for 30 min at 45°C. The mixture was added to 50 μl of P11 phosphocellulose (Whatmann) equilibrated with buffer B (10 mM Tris–HCl pH 7.5, 0.5 mM DTT, 0.2 mM AEBSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The mixture was rocked for 1 h at 4°C then phosphocellulose was recovered by centrifugation and washed with 1.5 ml of buffer B adjusted to 0.8 M NaCl. Contrary to the native enzyme, the dephosphorylated protein still binds phosphocellulose at this high ionic strength. After this treatment, phosphocellulose was equilibrated with 30 volumes of phosphorylation buffer (50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl) and incubated at 30°C for 30 min with 0.4 mU of CKII (Calbiochem) in a reaction mixture containing 50 μM ATP.

Construction of tagged DNA ligase I mutants

DNA ligase I mutants were obtained with suitable primers and PCR-mediated mutagenesis of the pLigI-Tag plasmid (Montecucco *et al.*, 1995b) as described previously (Montecucco *et al.*, 1998). Plasmids were analysed by restriction mapping and DNA sequencing (Thermo Sequenase kit, Amersham). All the oligonucleotides were purchased from Tib.Mol.Biol. (Genova, Italy). Expression of an appropriately sized fusion protein was verified by immunoblotting analysis of extracts from transfected cells with the anti-tag HUC1-1 mAb (ICN).

Preparation of DNA–protein complexes

DNA–protein complexes with a density of 1.4 g/ml, equivalent to that of native chromatin, were purified as described by Gohring and Fackelmayer (1997) from 3×10^7 exponentially growing HeLa cells. Cell extracts were prepared in the presence of 5 mM EDTA and 1 mM sodium orthovanadate to prevent dephosphorylation of hLigI.

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