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# Skp2 induction and phosphorylation is associated with the late G1 phase of proliferating rat hepatocytes

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**Abstract The changes in phosphoproteins purified with the affinity peptide p9CKShs1 were analyzed from extracts of regenerating rat livers in order to define some G1 and G1/S regulations characteristic of mature hepatocytes stimulated to proliferate. We observed a 47 kDa phosphoprotein that occurred first at the end of G1 before peaking in the S phase. P47 was also found to be phosphorylated in late G1 in primary hepatocyte cultures stimulated with mitogens. P47 was still phosphorylated in extracts depleted of Cdc2, but to a lesser extent after Cdk2 depletion. This phosphoprotein was identified as Skp2. (i) P47 shared the same electrophoretic mobility than Skp2, a cell cycle protein essential for S phase entry in human fibroblasts; (ii) Skp2, like P47, started to be expressed and was highly phosphorylated during the G1/S transition of hepatocytes stimulated to proliferate in vivo and in vitro; (iii) P47 was specifically immunoprecipitated by an antibody directed against Skp2. In addition, cyclin A/Cdk2 complexes from regenerating liver clearly interacted with Skp2. This is the first demonstration that Skp2 is induced and phosphorylated in the late G1 and S phase of hepatocytes in vivo in regenerating liver as well as in vitro in mitogen-stimulated hepatocytes.**



*Key words:* Cell cycle; G1/S transition; Skp2; Hepatocyte; Liver regeneration

## 1. Introduction

Normal hepatocytes are usually quiescent cells in vivo, but in contrast to most other cell types, they retain the ability to synchronously get into the active part of the cell cycle after liver cell loss. This is exemplified by the regeneration process following partial hepatectomy (PHT) [1]. This model is characterized by a particularly long G1 phase that can be analyzed and dissected into distinct steps. Immediately after PHT, hepatocytes enter in a state of pre-replicative competence before they can fully respond to growth factor. This priming step is an initiating event presumably induced by TNF-α and other cytokines mainly IL-1 and IL-6 [2–4]. Then, these 'initiated cells' require a growth factor stimulation to progress further in the late G1 and S phase. Different growth factors and hormones (HGF, EGF, TGF-α, insulin, norepinephrine) appear to play an important role in this regenerating process [5–9]. This process can be mimicked in vitro. Both EGF and HGF have been shown to stimulate signal transduction and MAP kinase cascade activity in rat hepatocytes in vitro [10–14]. A growth factor-dependent restriction point has been localized in vitro in mid-late G1 in primary rat hepatocytes [15]. These two models give us the opportunity to look at the events occurring during the G1 phase progression of highly differentiated hepatocytes.

The eukaryotic cell cycle molecular machinery is based on protein complexes composed of regulatory subunits (cyclins), catalytic subunits (Cdks for cyclin-dependent kinases) and some associated proteins [16–19]. Major elements in checkpoint controls acting at the G1 progression and G1/S transition are kinases and phosphatases that determine the state of phosphorylation of the Cdk/cyclin complexes (for review see [20]). The mechanisms of regulation that specifically control progression from the priming step to the late G1 phase in hepatocytes remain poorly elucidated. In this work, we used the strong affinity of the Cdks for the peptide p9CKShs1 (p9), allowing to purify the Cdks and Cdk-associated proteins in non-denaturing conditions in order to isolate the proteins interacting with these complexes in hepatocytes. This peptide is the mammalian homologue of CKS1 from *Saccharomyces cerevisiae* and p13suc1 from *Schizosaccharomyces pombe*, which has been used extensively to purify Cdc2, Cdk2 and their associated proteins [21,22].

We describe here a protein of 47 kDa (P47), which phosphorylation occurred in the late G1 and G1/S phase transition of the hepatocyte cell cycle. Analysis of its biochemical characteristics led us to further define the molecule and to question whether it could correspond to the Skp2 phosphoprotein firstly described by Zhang et al. in fibroblasts [19] but never reported in highly differentiated normal cells such as hepatocytes.

## 2. Materials and methods

### 2.1. Chemicals

[γ-$^{32}$P]ATP (3000 Ci/mmol), [$^{32}$P]$H_3PO_4$ (10 mCi/ml) and methyl-[$^3$H]thymidine (5 Ci/mmol) were from Amersham (Rainham, UK); protein A-Sepharose CL-4B, sephacryl S-100-HR and CNBr-activated Sepharose 4B were from Pharmacia; recombinant human EGF was from Promega (Madison, WI, USA). Bead buffer and buffer C were as described [23].

### 2.2. Antibodies

Anti-p34Cdc2 and anti-p33Cdk2 are rabbit polyclonal antiserum directed against C-terminal portions of human p34Cdc2 and p33Cdk2 [23]. Anti-human cyclin A antibodies raised against the full length cyclin A are obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two antibodies (Santa Cruz, CA, USA),

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*Abbreviations:* Cdk, cyclin-dependent kinase; PHT, partial hepatectomy; EGF, epidermal growth factor; MEM, minimum essential medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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anti-Skp2 p45 goat polyclonal antiserum against the carboxy-terminus of the human Skp2 and anti-Skp2 against the full lenght molecule, were used for immunoblotting and immunoprecipitation of translated products, respectively.

*2.3. Animals and cell cultures*

PHT (two-thirds) was performed according to the method of Higgins and Anderson [1] in accordance to French laws and regulation. Hepatocytes were isolated from rat livers by the two-step perfusion procedure [24]. Cells were plated in minimum essential medium (MEM)-199 (3:1) (v/v), added with 10% fetal calf serum (FCS). 4 h later and every day, it was renewed by a medium without FCS, supplemented with EGF (50 ng/ml) and sodium pyruvate (10 mM). NIH-3T3, FAZA 967, FAO were cultured in a mixture of Ham F12/NCTC 199 (1:1). Immortalized epithelial liver cells (RLEC) and HTC hepatoma cells were cultured in Williams medium and DMEM, respectively. All media were supplemented with 10% FCS.

*2.4. [$^3$H]Thymidine incorporation*

The rate of DNA synthesis was measured in animals following PHT or sham operation as described [23]. In hepatocyte cultures, 2 μCi of methyl-[$^3$H]thymidine was added for 24 h periods prior to cell harvesting at the indicated times. Incorporation was measured by scintillation counting after trichloroacetic acid precipitation.

*2.5. Cdk-cyclin complexes preparation and phosphorylation*

p9 was purified by gel filtration on sephacryl S-100 and coupled to CNBr-activated Sepharose 4B [25]. Briefly, protein extracts (100 μg) were bound to p9 beads for 60 min at 4°C, pelleted and washed [23]. Thereafter, p9 bead pellets were resuspended in 50 μl of C phosphorylation buffer containing 10 μCi [γ-$^{32}$P]ATP for 1 h at 30°C. The reaction was stopped by 60 μl of sample buffer and proteins were separated on 10% SDS-PAGE [26]. To identify alkali resistant phosphoproteins, the gels were soaked in 2 M NaOH for 1 h at 55°C [27] prior to drying and autoradiography. The phosphoamino acid was analyzed on cellulose thin layer plates as previously described [28].

*2.6. [$^{32}$P]$H_3PO_4$ incorporation*

At indicated times, hepatocytes were incubated for 1 h in culture medium containing 250 μCi [$^{32}$P]$H_3PO_4$. Then, the proteins were bound to p9 beads as described above.

*2.7. Immunoblotting and immunoprecipitations.*

After SDS-PAGE, proteins were transferred onto nitrocellulose membranes according to established protocols. Immunoprecipitations were performed for 2 h at room temperature in the presence of protein A-Sepharose. For immunodepletions, the supernatant was re-immunoprecipitated four times. The final supernatants were bound to p9 beads and phosphorylated as described above.

*2.8. cDNA cloning, reverse transcription (RT)-PCR and Northern blotting analyses*

Rat Skp2 cDNA probe was obtained by RT-PCR based on the human sequence data [19] using primers 5′-TAGACAACTGGGCTTTTGCAGAGTC-3′ and 5′-CTTCCGGATGAGCTGCTCTTGGGAA-3′ from HTC cells.

Rat Skp2 PCR product was cloned and DNA sequencing was performed. Primers 5′-CTGCAGAATCTGAGTCTGGAAGGC-3′ and 5′-TAGTGTGGGGATTTCTCCGAGTTC-3′ specific for rat Skp2 were used for direct semi-quantitative Skp2 RT-PCR analysis. Total RNA extracted from hepatocytes stimulated or not by EGF/pyruvate and from liver cell lines were used for Northern blotting as described previously [15].

The quality of cDNA was confirmed by PCR amplification of a similar-sized fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

*2.9. Binding assays*

Cdk2 and cyclin A immunoprecipitations were performed by incubating 0.5 mg of 24 h liver extract in binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM EDTA, 1 mM dithiothreitol and 5 μg/ml aprotinin, leupeptin and soybean trypsin) with specific antibody at 4°C overnight and then, in the presence of protein A-Sepharose for 1 h at 4°C. The samples were then pelleted and washed three times. Human full length Skp2 cDNA was obtained using primers 5′-TAGACAACTGGGCTTTTGCAGAGTC-3′ and 5′-GCAGCTCTGCAAGTTTAATGCACGT-3′. [$^{35}$S]methionine-labelled full length human Skp2 (Skp2 (wt)), was produced in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. A clone of Skp2 with a stop codon at positions 630–632, determined from the translation initiation site, was used to generate a C-terminal truncated form (Skp2 (ΔC)). 10 μl translation product was mixed with Cdk2 or cyclin A immunoprecipitates, diluted to 150 μl with binding buffer. The mixture was incubated on ice for 1 h. Beads were washed three times, then, separated on a 15% SDS-polyacrylamide gel, fixed, dried and subjected to autoradiography.

## 3. Results

*3.1. P47 phosphoprotein is observed in the late G1 phase of regenerating liver*

[$^3$H]Thymidine incorporation was measured at different times after PHT allowing to define the G1 and S phases of the cell cycle in this model. Fig. 1A shows that the DNA synthesis starts at 20 h after surgery and peaked at around 24 h. This localizes the G1/S transition of these cells 18–20 h following PHT.

In order to select proteins associated with Cdks/cyclins dur-

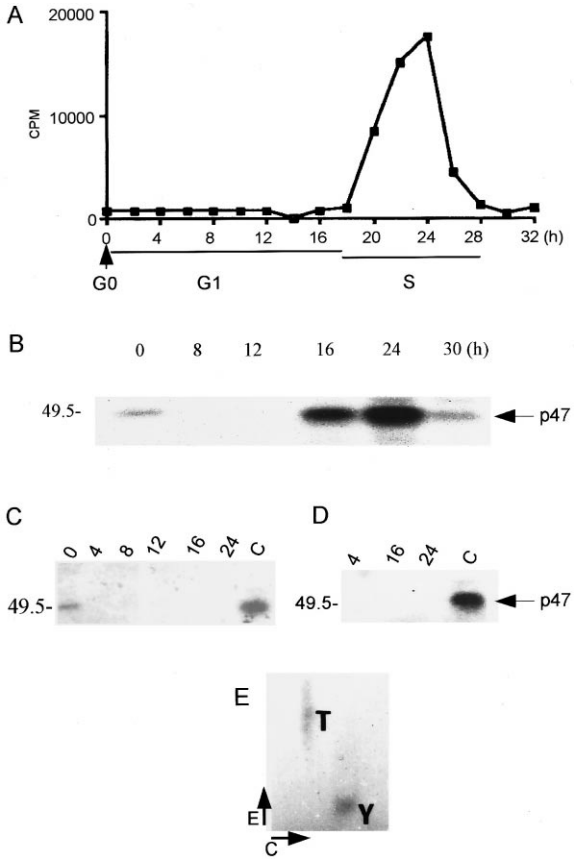


Fig. 1. Phosphorylation of p9CKShs1-associated protein complexes extracted from regenerating rat livers. (A) Time course of [$^3$H]thymidine incorporation into DNA of regenerating liver following PHT. (B) Alkaline resistant phosphoprotein patterns of proteins purified by binding to p9(CKShs1) beads at the indicated times. (C) p9 minus beads-associated alkaline resistant phosphoproteins from regenerating rat liver. (D) p9 bead-associated phosphoproteins from non-regenerative liver (sham) at the indicated times after surgery: P47 from 24 h regenerating liver purified with p9 beads as a positive control (C). (E) Phosphoamino acid analysis of P47. Phosphoamino acids were analyzed by electrophoresis (E) followed by chromatography (C). T, phosphothreonine; Y, phosphotyrosine.

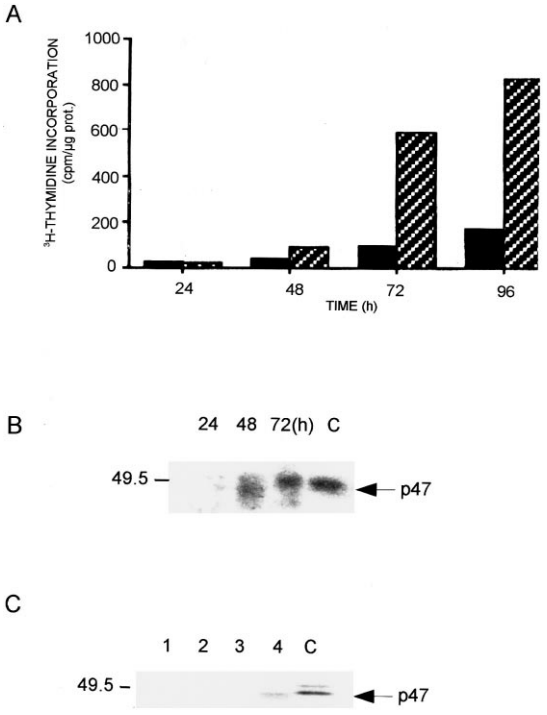


Fig. 2. Phosphorylation of p9CKShs1-associated protein complexes from mitogen-stimulated primary rat hepatocyte cultures. (A) Time course of [$^3$H]thymidine incorporation into DNA of hepatocytes stimulated (hatched) or not (black) with EGF/pyruvate. (B) Alkaline resistant phosphorylation proteins purified with p9 beads and extracted from mitogen-stimulated cultures at the indicated times after seeding. (C) [$^{32}$P]Orthophosphate-labelled proteins purified with p9 beads from 24 h (1, 2) and 48 h (3, 4) hepatocyte cultures stimulated (2, 4) or not (1, 3) with EGF (C, P47 control from 24 h regenerating liver).

ing the G1 progression and S phase of regenerating livers, we purified them by coupling tissue extracts from liver, at different times after PHT, with the small peptide p9(CKShs1)-coated beads. The proteins were phosphorylated in vitro with [$\gamma$-$^{32}$P]ATP at 30°C for 60 min. We chose to perform alkaline digestion on SDS-PAGE gels because of the high frequency of cell cycle-associated proteins to be phosphorylated onto threonine and tyrosine residues. Indeed, phosphotyrosine as well phosphothreonine but not phosphoserine residues were resistant to a different degree to alkaline exposure [27]. Among phosphoproteins, only a few were found to resist alkaline digestion: a 47 kDa protein (P47) was strongly phosphorylated and a 52 kDa protein to a lesser extent (Fig. 1B). These proteins were first identified at the end of G1 and peaked in the middle of the S phase. A 49 kDa phosphoprotein was also present in the normal liver but disappeared very soon after PHT.

The specificity of these phosphoproteins regarding p9 binding was established by analyzing the pattern of phosphoproteins bound to pure Sepharose beads. The 49 kDa phosphoprotein from normal liver was found to be phosphorylated revealing its unspecific binding to p9 beads (Fig. 1C), whereas P47 phosphoprotein could not be seen. In addition, extracts from non-regenerating liver from sham-operated animals bound to p9 beads did not show any alkaline resistant phosphoprotein (Fig. 1D).

To evidence threonine and tyrosine phosphorylation sites of p47, we performed amino acid analysis of this alkaline resistant phosphoprotein by excising the band from the SDS-PAGE gel, hydrolyzing with HCl and resolving it on two-dimensional cellulose thin layer plates. We found that P47 was phosphorylated on two residues (Fig. 1E).

### 3.2. P47 phosphorylation also occurs in late G1 in mitogen-stimulated hepatocyte primary cultures

We previously showed that rat hepatocytes in primary cultures replicated their DNA after EGF/sodium pyruvate stimulation [15]. In continuous presence of the mitogen, the cells entered the S phase 48–50 h after seeding and the peak of DNA synthesis occurred at 96 h (Fig. 2A). A phosphoprotein bound to p9 beads and co-migrating with P47 was detected in these cells, first at 48 h in the late G1 phase and accumulated thereafter in the S phase (Fig. 2B). Furthermore, phosphorylation activity was assessed by incubating hepatocyte cultures in the presence of [$^{32}$P]$H_3PO_4$. A p9-bound phosphoprotein similar to P47 also appeared at 48 h of culture in the mitogen-stimulated hepatocytes but not in control unstimulated cells (Fig. 2C). Our results show that P47 is phosphorylated in vivo as well as in vitro in the late G1 phase of proliferating hepatocytes.

In order to look for the presence of P47 in other cell types

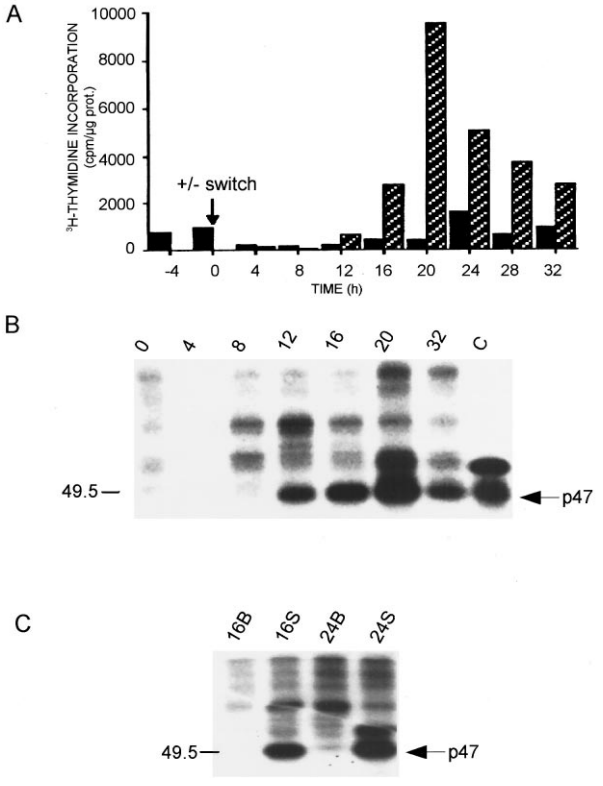


Fig. 3. Phosphorylation of G1-synchronized NIH-3T3 mouse fibroblasts. (A) Time course of [$^3$H]thymidine incorporation into DNA of NIH-3T3 cells synchronized for 24 h with 10 mM sodium butyrate and kept blocked (black) or switched at time 0 to 10% FCS (hatched) in order to stimulate cell cycle progression. (B) Alkaline resistant phosphoproteins from p9-purified proteins extracted from NIH-3T3 cells at the indicated times following release of the butyrate blockage (c, control from 24 h regenerating liver extract). (C) Alkaline resistant phosphoproteins from NIH-3T3 cells maintained blocked (16B, 24B) in butyrate or released in FCS (16S, 24S) at 16 and 24 h following medium change.

in vitro, we chose to block NIH-3T3 mouse fibroblasts in early G1 by sodium butyrate (10 mM) and serum deprivation and to analyze the G1 and S phase progression of these cells after release of this block by 10% FCS addition. The G1/S transition occurred 12/16 h after the release and was followed by a well-synchronized S phase (Fig. 3A). Several phosphoproteins were found to resist alkali treatment and a phosphoprotein co-migrating with the rat liver P47 was also detected in NIH-3T3 synchronized fibroblasts (Fig. 3B). Phosphorylation of the protein appeared in the late G1 phase (12 h), peaked in the S phase (20 h) and decreased thereafter. The cells that remained blocked in the early G1 phase in the presence of butyrate never showed any evidence of P47 phosphorylation (Fig. 3C).

### 3.3. P47 could interact with Cdk2

Since p9 is known to bind different cell cycle proteins, we looked if there was any relation between p47 and some well-characterized kinases, i.e. Cdc2 and Cdk2. These protein kinases have been shown to be expressed in rat hepatocytes in relation to the cell cycle and their histone H1 kinase activity was found in the S phase [15].

Therefore, we performed selective immunodepletions of each protein to determine if these protein kinases were part of a complex to which phosphorylated P47 could be associated. We first cleared Cdc2 or Cdk2 from tissue extracts as evidenced by Western blotting (Fig. 4A, B) and then, we performed p9 binding. We showed that phosphorylation of P47 bound to p9 was not significantly modified in Cdc2-depleted extracts (Fig. 4C) while it was significantly decreased after Cdk2 depletion, indicating that this kinase could interact with P47.

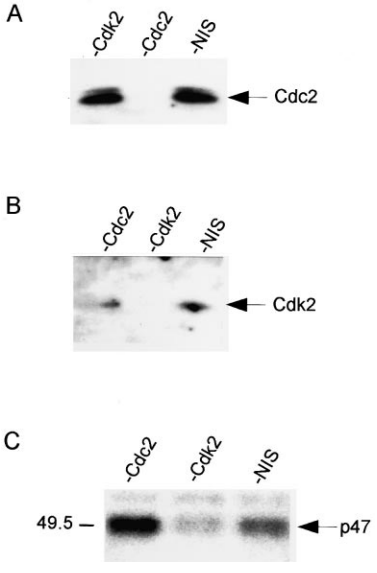


Fig. 4. Phosphorylation of P47 from Cdc2- and Cdk2-immunodepleted 24 h regenerating liver (-Cdc2, -Cdk2). (A) Anti-Cdc2 and (B) anti-Cdk2 Western blots of Cdc2- and Cdk2-immunodepleted 24 h regenerating liver extracts. (C) Phosphorylation of p9-associated proteins following immunodepletion of Cdc2 and Cdk2 (NIS, control depletion with a non-immune serum).

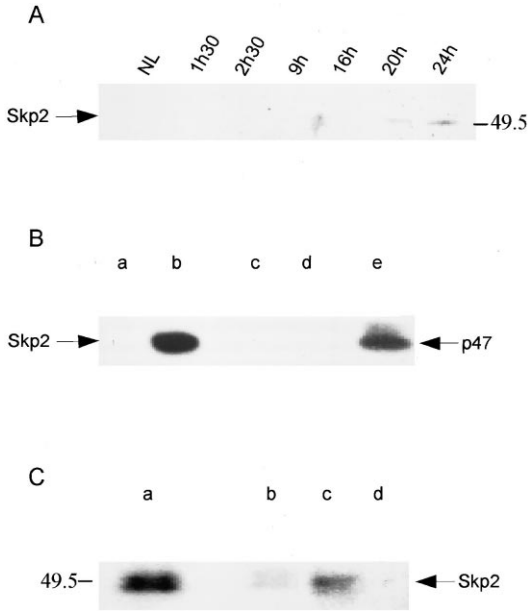


Fig. 5. Skp2 protein expression and phosphorylation in regenerating liver. (A) Western blotting analysis of Skp2 in regenerating liver at the indicated times after PHT. (B) Alkaline resistant phosphorylation after immunoprecipitation by Skp2 antiserum at 14 h (a) and 24 h (b) following PHT. Controls from non-immune serum at the same times (c, d) and P47 from the 24 h regenerating liver (e). (C) Immunoprecipitation of P47 by Skp2 antiserum. P47 from 24 h regenerating liver (a). P47 was solubilized by a mixture of non-denaturant detergent and the residual P47 on p9 beads was analyzed (b). Then, the solubilized P47 was immunoprecipitated by Skp2 antiserum (c) or a non-immune serum (d).

### 3.4. Skp2 is induced, phosphorylated and interacting with the Cdk2/cyclin A complex upon S transition of regenerating liver in vivo

Skp2 has been described to occur in the S phase of normal and transformed fibroblasts as a phosphoprotein of 45 kDa, which interacts with the Cdk2/cyclin A complex [19]. In order to see whether P47 could be Skp2, we looked first at the appearance of Skp2 in regenerating liver and compared its electrophoretic mobility to that of P47. Then, we deviced experiments to analyze the ability of the anti-Skp2 antibody to immunoprecipitate P47. Then, we looked at the interactions between Skp2, cyclin A and Cdk2 in 24 h regenerating liver.

As shown in Fig. 5A, the Skp2 expression kinetics were identical to that of P47 phosphorylation, it started to be detected 20 h after PHT at a time corresponding to the G1/S transition. Thereafter, the protein level increased in the S phase, i.e. 24 h after PHT. In addition, Skp2 immunoprecipitation from cell extracts 4 and 24 h after PHT revealed one alkaline resistant protein in the 24 h extract sharing the same electrophoretic mobility as P47 (Fig. 5B). As expected, no Skp2 phosphorylation could be detected with a non-immune serum.

Conversely, P47-phosphorylated protein bound to p9 beads from 24 h PHT extracts was solubilized in a mixture of detergent (TX100 1%, DOC 1%, SDS 0,01%) and immunoprecipitated with Skp2 antibody. As shown in Fig. 5C, a characteristic P47 band was evidenced with Skp2 antibody while no band could be detected with a non-immune serum.

Finally, Cdk2/cyclin A complex interaction with Skp2 was demonstrated in regenerating rat liver. Two human Skp2

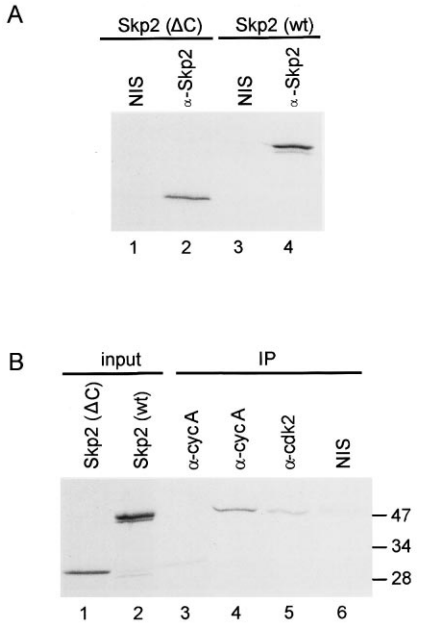


Fig. 6. Liver Cdk2/cyclin A S phase interaction with Skp2. (A) Immunoprecipitation of translated products of truncated Skp2 (ΔC) (1, 2) or full length Skp2 (wt) (3, 4) by a polyclonal antibody against human Skp2 (2, 4). Controls with a non-immune serum (1, 3). (B) Binding of [$^{35}$S]-labelled truncated (3) or full length (4–6) Skp2 proteins to cyclin A (3, 4), Cdk2 (5) from 24 h regenerating liver extracts and to control extract with a non-immune serum (6). Electrophoretic migration of Skp2 (ΔC) (1) and Skp2 (wt) (2) as control (input).

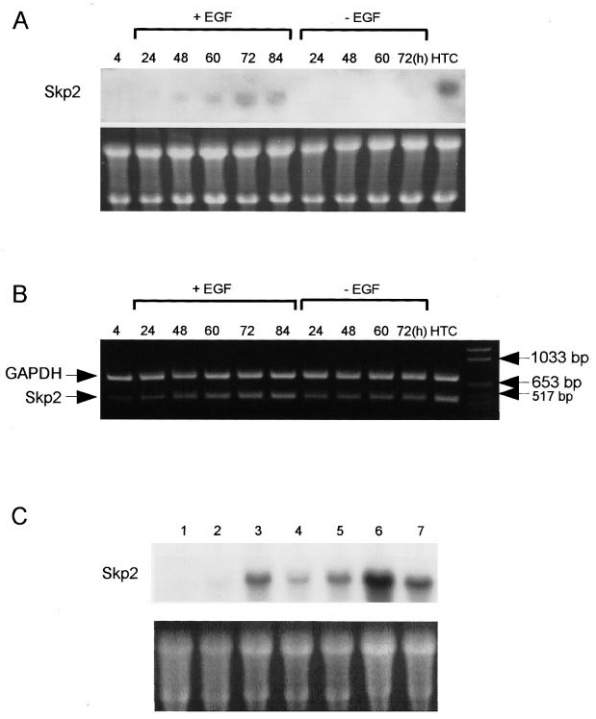


Fig. 7. Skp2 Northern blotting (A, C) and RT-PCR analysis (B). Total RNA was extracted at the indicated times from a primary culture of hepatocytes stimulated (+EGF) or not (−EGF) by growth factor 4 h after seeding. Skp2 Northern blotting (A) and RT-PCR (B) analysis. Control: PCR amplification of a similar-sized fragment of GAPDH. (C) Skp2 Northern blotting analysis of total RNA extracted from a 4 h (1) and 48 h (2) unstimulated primary culture of hepatocytes, proliferative hepatoma cells HTC (3), FAZA (4), FAO (5) and immortalized liver epithelial cells (RLEC) in a proliferative state (6) and at confluency (7).

cDNA probes obtained by RT-PCR and corresponding to the full length protein and a truncated form of the molecule were selected. These clones were translated in a reticulocyte lysate system in the presence of [$^{35}$S]methionine and immunoprecipitated by specific polyclonal antibody against the full length Skp2 of human origin (Fig. 6A). This polyclonal antibody recognized two proteins corresponding to the expected sizes.

Then, we performed immunoprecipitations of 24 h regenerating liver extracts using specific antibodies against Cdk2 or cyclin A. Immunoprecipitates were incubated with the [$^{35}$S]-labelled Skp2 proteins, washed and analyzed by SDS-PAGE. As shown in Fig. 6B, cyclin A and Cdk2 complexes were able to bind the full length Skp2 protein but not the truncated form. No protein could be detected with a non-immune serum.

### *3.5. Skp2 mRNA expression is induced in proliferating hepatocytes in vitro and hepatoma cells*

To provide additional evidence on the expression kinetics of Skp2 in growth-stimulated hepatocytes, first, we looked at the Skp2 mRNA expression in a primary culture of hepatocytes stimulated by EGF by using Northern blotting. As shown in Fig. 7A, Skp2 mRNA was expressed in EGF-stimulated cells according to their progression in the G1 phase while non-stimulated hepatocytes did not show a detectable level of Skp2 mRNA. Secondly, semi-quantitative RT-PCR has allowed to evidence an increased Skp2 PCR product amplification in proliferating hepatocytes while a low level was observed in unstimulated cell cultures (Fig. 7B).

Since Skp2 is known to greatly increase in many transformed cells [19,29], we looked at its expression in different immortalized (RLEC) and transformed (HTC, FAZA, FAO) liver cell lines (Fig. 7C). Skp2 mRNA was highly expressed in all the actively growing hepatoma cell lines analyzed, although differences in the steady state levels were observed from one line to the other. Furthermore, proliferating immortalized rat liver epithelial cells (RLEC) appeared to express a higher level of Skp2 mRNA than the resting cells at confluency, showing that Skp2 expression was also correlated with a growing activity in these cells.

## 4. Discussion

The approach used in this study allowed us to pinpoint a phosphoprotein associated to the proteins, which controls the cell cycle at the G1/S transition of proliferating hepatocytes. We identified this phosphoprotein as Skp2 and showed for the first time that this protein was induced and phosphorylated in vivo in the late G1 and S phase of regenerating liver as well as in vitro in mitogen-stimulated normal hepatocytes.

It has been established for a long time that cyclins, Cdks and associated proteins, which are actors of prime importance in the cell cycle, are regulated through phosphorylation changes of some of their residues. An increased level of cyclin D1, in mid-late G1, correlates with cell cycle progression through the mitogen-associated restriction point. In late G1 and S phase, Cdc2 and Cdk2 kinases are activated in association to cyclins A, B and E [15,23,30,31]. All proteins inter-

acting with these complexes are not well-identified in hepatocyte and regulation mechanisms responsible for the ability of these growth-arrested differentiated cells to duplicate following a growth signal remain poorly understood. The objective of our study was to look at changes in phosphorylation of the cell cycle proteins in order to identify some candidates for the regulation of G1 progression and G1/S transition of proliferating hepatocytes. The use of p9CKShs1, in non-denaturing conditions, enabled us to purify Cdk/cyclin complexes and their associated proteins [22]. We focused our attention on proteins phosphorylated on both threonine and tyrosine by alkaline lysis of phosphoserine.

This strategy has allowed us to detect only a few alkaline resistant proteins associated with p9 beads and among them, a P47 phosphorylation started to be observed at the end of G1, just prior to the S phase, where it reached a maximum. The phosphorylated protein was specifically associated with the cell replication in growth-stimulated hepatocytes as shown by its kinetics which are strictly correlating with cell progression through the G1 and S phase, using two different model systems: regenerating rat liver and hepatocyte primary cultures stimulated by a growth factor. Furthermore, by detecting P47 at the end of G1 and peaking in the S phase of synchronized NIH-3T3, we extend to non-hepatic cells the expression and regulation of this cell cycle-associated protein. Because of its molecular weight, we investigated whether P47 could be cyclin E or a MAP kinase (ERK 1 or ERK 2) using specific antibodies and we ruled out these possibilities. In contrast, interestingly, we identified P47 as Skp2, a 45 kDa protein in human cells. Several characteristics argue for Skp2 identification:

(i) The kinetics of Skp2 expression and phosphorylation in hepatocytes were similar to those of P47 phosphorylation.

(ii) Similar characteristics in molecular weight and in sites of phosphorylation on tyrosine and threonine residues in late G1 were observed.

(iii) The ability of Skp2 antibody to recognize one protein bound to a p9 bead and resistant to alkali treatment namely P47 was clearly established.

Overall, from these data, evidence is provided for the first time that Skp2 is expressed in normal adult hepatocytes and its transcriptional regulation is strictly associated to the cell cycle progression, its induction being located just before the G1/S transition. Noteworthy, the fact that changes in the phosphorylation status of the protein followed the same kinetics provides strong arguments in favor of a crucial role of this molecule in the cell decision to undergo DNA synthesis.

It is important to note that P47 was also identified at a time corresponding to the G1/S transition in $[^{32}P]H_3PO_4$-labelled hepatocytes stimulated by EGF, providing confirmation that this protein was phosphorylated in late G1 in proliferating hepatocytes in situ prior to any interactions with exogenous p9 protein.

Skp2 was initially identified in transformed cells and in a recent study, overexpression was found in 17 of 31 (55%) human hepatocellular carcinomas [32]. Here, we also confirm an overexpression of Skp2 mRNA in several hepatoma cell lines. In addition, an expression associated with the proliferation is suggested in immortalized cells like RLEC.

Zhang et al. have characterized two proteins of 19 and 45 kDa (SKp1 and Skp2) that are essential elements of the Cdk2/cyclin A S phase kinase in human fibroblasts [19]. Furthermore, while Skp2 can inhibit the kinase activity of Cdk2/cyclin A toward histone H1, Skp2 itself appears to serve as a substrate at the same time. Recently, Ser-76 was found to be the major site of Skp2 phosphorylation by Cdk2/cyclin A [29]. In this report, we showed that proteins of the Cdk2/cyclin A S phase complex of regenerating liver clearly interact with Skp2. Moreover, immunodepletion of Cdk2 also evidenced that Skp2 phosphorylation was, at least partly, dependent on the presence of Cdk2, indicating that Cdk2 is one of the kinases interacting with the Skp2 in differentiated liver cells stimulated to proliferate as it was described in dividing human fibroblast and HeLa cells [19,29]. Untill now, Skp2 phosphorylation onto other sites than serine has not been reported. Using an alkali resistant gel, we demonstrate here that Skp2 could also be phosphorylated on a threonine and tyrosine and that this phosphorylation activity was strictly associated with the cell cycle.

While the role of Skp2 in promoting the S phase is well-established, the mechanism involved in this process is not yet completely understood. Skp2 could be associated to the ubiquitination machinery process by its F box domain interactions. Indeed, recently, Lisztwan et al. have proposed that, to promote the S phase, eukaryotic cells might use an ubiquitin conjugation apparatus governed in part by periodic S phase-specific accumulation of the Skp2 subunit [33]. Skp2 belongs to the family of F box proteins which play a critical role in ubiquitin-mediated protein degradation in *S. cerevisiae* [34]. Moreover, the human Skp2/SKp1/Cul-1 complex is likely to function as an E3 ligase to selectively target cyclin D and P21 for ubiquitin-dependent protein degradation [35]. Our study pinpoints Skp2 as a potential player in the G1/S transition and S phase of mature hepatocytes and, for the first time, shows that Skp2 is regulated in a cell cycle-dependent manner in normal highly differentiated cells in vivo as well as in vitro. Its role in the complex control machinery of proliferating hepatocytes is presently under investigation.

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