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Induction of stathmin expression during liver regeneration

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Stathmin is a 19 kDa cytoplasmic phosphoprotein proposed to act as a relay for signals activating diverse intracellular regulatory pathways. After two-thirds partial hepatectomy, the concentration of stathmin reached a peak between 48 and 72 hours, comparable to the levels observed in neonatal liver, at about 10 times the basal adult level. Stathmin then decreased to basal levels within 7 days, more rapidly than during postnatal tissue development (7 weeks), with no detectable change in its phosphorylation state. Interestingly, the mRNA for stathmin reached a peak much earlier than the protein, at 24 hours posthepatectomy, and decreased to a still detectable level until 96 hours after hepatectomy. Altogether, the present results further support the generatility of the implication of stathmin in regulatory pathways of cell proliferation and differentation during normal tissue development and posttraumatic regeneration.

Stathmin; Cell proliferation; Hepatectomy; Tissue regeneration; Development

1. INTRODUCTION

During development and tissue regeneration, cell proliferation and differentiation are controlled by numerous extracellular and intracellular signals. Stathmin, also designated p19 [1], pp17 or prosolin [2], p18 [3], pp20 [4] and 19K [5], was recently identified as an ubiquitous phosphoprotein proposed to act as an intracellular relay integrating diverse second messenger signals triggered by various extracellular agents [6,7]. Indeed, phosphorylation of stathmin could be related in various instances to the regulations of cell proliferation [2,8,9], differentiation [10] and functions [1,11–14]. It is widely distributed in tissues and cell types [6,15], its expression is developmentally regulated [15–17], and its structure as well as its sequence is phylogenetically highly conserved [15,18].

Liver regeneration after partial hepatectomy is widely used to study cell and tissue growth regulation, as well as cell cycle-related events in vivo. Following two-thirds hepatectomy, the normally quiescent hepatocytes of the adult rat liver undergo a partially synchronous wave of DNA replication followed by cell division [19]. The rapid initiation as well as termination of constitutive DNA synthesis in hepatocytes after hepatectomy are precisely coordinated. Most hepatocytes in the remaining liver lobes divide once or twice following two-thirds hepatectomy so that the number of hepatocytes can be returned to normal [19,20]. Thus, liver regeneration is a particularly suitable system, in the whole animal and adult solid tissue, to investigate various functions regarding the transition from quiescence to proliferation and the cell cycle progression [21–25].

To better characterize the implication and roles of stathmin in regulations of the cell proliferation and maturation events underlying normal tissue development and posttraumatic regeneration, we examined the time-course of stathmin mRNA and protein expression as well as the distribution of its unphosphorylated and phosphorylated forms in livers during (i) postnatal development (0–7 weeks) and (ii) liver regeneration after partial hepatectomy (0–14 days). We show that stathmin expression increases considerably during hepatectomic regeneration, without detectable changes in its phosphorylation state, with a peak of mRNA at 24 h followed by a peak of protein expression only 24–48 h later. Stathmin then returns to basal levels in a way comparable to, but faster than its postnatal developmental pattern. The present results thus further support the proposed general role of stathmin in cell regulations, in a physiological model of tissue development and regeneration.

2. MATERIALS AND METHODS

2.1. *Materials*

Seven-week-old male Wistar rats (250 g) from CERJ (France) and female Sprague–Dawley rats (200 g) from Charles River (France) were used for long-term and short-term hepatectomy experiments, respectively. For developmental studies, livers were removed from Wistar rats at different ages. Chemicals and their sources were: leupeptin, aprotinin, pepstatin (Sigma), reagents for polyacrylamide gels (Serva, Bio-Rad and Sigma), ampholines (Pharmacia), casein (Serva),

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nitrocellulose 0.2 μm (Schleicher and Schuell), Nylon N^+ transfer membrane and [^{125}I]protein A (Amersham).

2.2. *Partial hepatectomy*

Two-thirds partial hepatectomy was performed according to the method of Higgins and Anderson [26], sham-operations being performed as controls. For the long-term experiment (12 h–14 days), groups of 3–4 animals were sacrificed and their livers removed at indicated times. For the short term experiment (12–120 h), rats were killed at indicated times and their livers were perfused with PBS for 10 s. The livers were quickly frozen in liquid nitrogen and kept at −80°C.

2.3 *Tissue extracts*

Frozen livers were homogenized in 6 volumes of homogenization buffer (Tris-HCl pH 7 4 10 mM, NaN_3 0.02%, leupeptin 10 μg/ml, aprotinin 25 μg/ml, pepstatin 10 μg/ml, EDTA 1 mM) in a Polytron homogenizer and centrifuged at 100,000 rpm for 6 min in a Beckman TL-100 centrifuge. The high-speed supernatant 'S2' was adjusted at 100 mM NaCl, incubated at 100°C for 2 min and centrifuged again. The resulting stathmin-enriched 'S3' supernatant [6] was used directly for one-dimensional (1D-PAGE) electrophoresis. For two-dimensional (2D-PAGE) analysis, the 'S3' supernatant was dialysed against 0.2 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM NaCl, to allow lyophilisation and further concentration of samples.

2.4. *Polyacrylamide gel electrophoresis*

Discontinuous polyacrylamide electrophoresis in 1D- and 2D-PAGE were performed as previously described [15] About 50 μg of protein in 'S3' preparation were analysed by both 1D- (immunoblot) and 2D- (silver staining according to [27]) PAGE. Protein concentrations were estimated by the method of Bradford [28] using BSA as a standard.

2.5. *Immunoblotting*

Electrotransfer of 1D-PAGE gels on nitrocellulose were performed with an LKB semi-dry blotting apparatus as previously described [15]. Stathmin immunoreactivity was detected with the rabbit polyclonal antiserum prepared against an internal peptide of stathmin (peptide I) used at a1:10.000 dilution for immunoblots [15], and revealed by [^{125}I]protein A and autoradiography. Quantification of immunoreactive bands on autoradiograms was performed with a Samba image analysis system (Grenoble, France), as described [29].

2.6. *RNA extraction and Northern blots*

Frozen biopsies (200 mg) were used to extract total RNA by the thiocyanate-guanidium procedure [30]. 20 μg of total RNA were resolved by electrophoresis in a 1.2% agarose gel containing 1.1 M formaldehyde, and transferred onto Nylon N^+ membrane. The prehybridized [31] blots were probed successively with multiprime labeled [32] fragments of rat stathmin [33] and albumin [34] cDNA. Stathmin hybridization was done first at 42°C in 50% formamide, 5 × SSC, 0.1% SDS and 2 × Denhardt's, with the last washes at 42°C in 0.2 × SSC, 0.1% SDS. The same blots were dehybridized in a 0.5% SDS boiling solution and rehybridized with the albumine probe as an internal control, with successive washes at 65°C in 3 × SSC, 1 × SSC and 0.1 × SSC with 0.1% SDS. Quantification of autoradiograms was performed as for immunoblots.

3. RESULTS AND DISCUSSION

3.1. *Stathmin expression in regenerating liver*

In several biological systems, stathmin expression was shown to be regulated by diverse molecular signals, in relation with embryonic and postnatal development and the regulation of cell proliferation and differentiation. Since liver regeneration after partial hepatectomy is widely used to study cell and tissue growth regulation in vivo, we examined the time course of stathmin expression in normal proliferating cells of regenerating compared to quiescent liver. The concentration of stathmin was determined as a function of time following two-thirds hepatectomy, by an immunoblotting assay after 1D-PAGE electrophoretic separation of the proteins from a stathmin-enriched, boiled liver extract, with a specific anti-stathmin antiserum recognizing a single 19 kDa band in liver extracts (Fig. 1). After hepatectomy the concentration of stathmin started to increase at 24 h, to reach a peak at about 10 times the basal level between 48 and 72 h; it then gradually returned to the level of normal liver, 7 days after hepatectomy (Figs. 1 and 2). No changes in stathmin concentration were observed after sham operation (Fig. 1).

Although our experimental design does not allow to exclude the possible involvement of nonparenchymal cells, it is most likely that the observed changes in stathmin concentration take place mostly in parenchymal

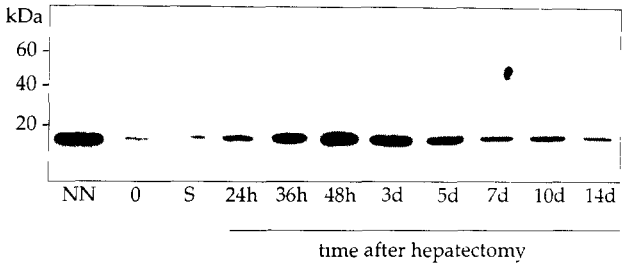

Fig. 1. Immunoblot detection of stathmin in neonatal rat liver and during hepatic regeneration. Stathmin-enriched, heat-soluble protein extracts were prepared from livers of neonatal (NN) and control and operated adult rats, (O) control, (S) 48 h after sham-operation, and at indicated times after partial hepatectomy (see section 2). The samples were electrophoresed on 1D-PAGE gels (50 μg protein/sample), transferred to nitrocellulose, and stathmin (19 kDa) was detected with a specific antiserum, [^{125}I]protein A and autoradiography.

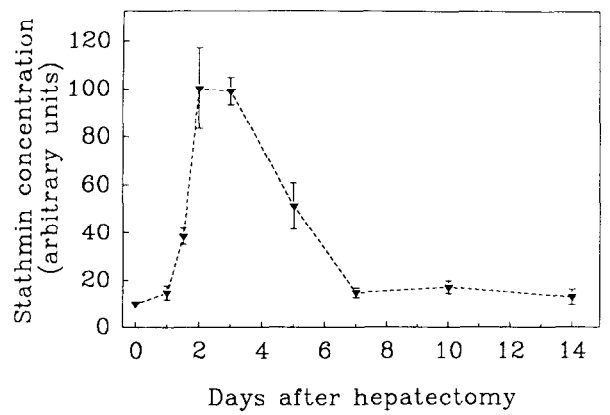

Fig. 2. Quantification of stathmin protein during hepatic regeneration up to 14 days after surgery. Stathmin was detected and quantified on 1D-PAGE immunoblots (see Fig. 1). Results from 3 4 samples for each point are expressed in arbitrary units (mean ± S.D.).

cells, since hepatocytes constitute the largest proportion (80–90%) of liver cell mass. Actually, hepatocytes in culture displayed a similar increase of stathmin expression on the third day after EGF stimulation (not shown), when cell division is at its maximum [35]. Furthermore, preliminary immunohistochemical results showing that stathmin is detectable in regenerating hepatocytes but not in quiescent ones (data not shown) also support this interpretation, in agreement with the observed expresion of stathmin mRNA in isolated hepatocytes 3 days after CCl_4-induced liver regeneration [36].

3.2. *Comparison of stathmin expression during development and after hepatectomy*

To assess the relevance of the changes in stathmin expression during liver regeneration to the physiological events taking place during development, we also examined its developmental expression pattern. Stathmin expression was the highest after birth and gradually declined towards adulthood. As described previously for mouse liver [15], we found that stathmin is 10 times more abundant in neonatal rat liver than in the adult (Figs. 1 and 3). Interestingly, expression of stathmin at its peak 2 days after hepatectomy was of the same order as the level observed in neonatal liver (Figs. 1 and 3). As revealed by immunoblot quantification, both levels then decreased to similar extents, although much more rapidly during posthepatectomic regeneration than during normal tissue development: approximately 10 fold decrease within 7 weeks after birth and 7-fold decrease in 7 days after hepatectomy (Fig. 3). Altogether, the

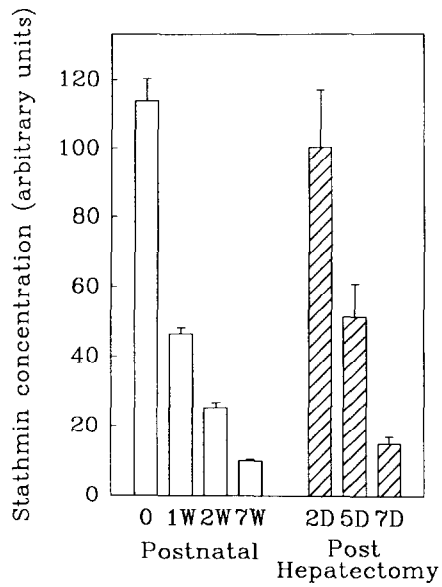

Fig. 3. Compared expression of stathmin during postnatal development and after hepatectomy. Stathmin-enriched, heat soluble protein extracts were prepared from rat livers removed at different ages (W: weeks postnatal) and processed similarly as described for Fig. 2 (left). Representative values of stathmin expression after hepatectomy on 7 week old rats are shown in parallel (D: days after hepatectomy) (right).

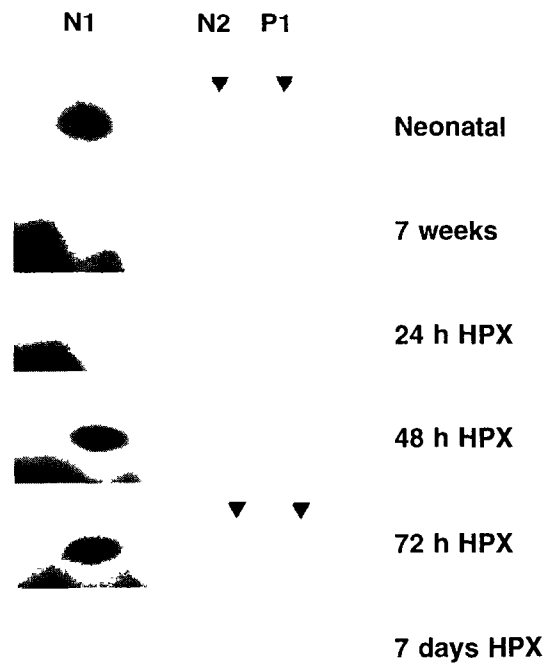

Fig. 4. 2D-PAGE patterns of stathmin isoforms after hepatectomy and during development. Stathmin-enriched, heat soluble protein extracts were prepared from rat livers at different ages and after partial hepatectomy (HPX), electrophoresed (50 μg protein/sample) on 2D-gels and silver stained. The areas corresponding to the non-phosphorylated (N1, N2) and phosphorylated (P1) forms of stathmin (19 kDa, pI 6.2–5.8) are shown. No additional pattern change was observed at two-hour intervals at intermediate times between 24 and 36 h posthepatectomy.

posthepatectomic regeneration model for liver development appears also as a faithful model for the involvement of stathmin expression.

Stathmin expression was shown previously to be developmentally regulated in various tissues and organs [15,37]. Stathmin is abundant in brain and testis, and its expression in vivo is not restrained to certain cell lineages but rather appears in a differentiation stage-dependent manner [38,39]. Stathmin is also highly expressed in multipotential cells of the inner cell mass of the mouse blastula, as well as in embryonic carcinoma cells in culture; its level decreases in differentiated embryonic layers and up to tenfold after retinoic acid and cAMP induced endodermal differentiation of F9 teratocarcinoma cells [29].

Kuo et al. [40] suggested that hepatocytes after birth until 8 weeks exhibit a relatively steady and differentiated pattern of gene expression (at least for albumin and transthyretin mRNA). Therefore it is possible that the reduced levels of stathmin expression observed in liver during postnatal growth are not associated directly with the differentiated state of hepatocytes, but rather with the termination of their proliferation and possibly differentiation processes. Thus, the observed changes of stathmin expression after hepatectomy could be inter-

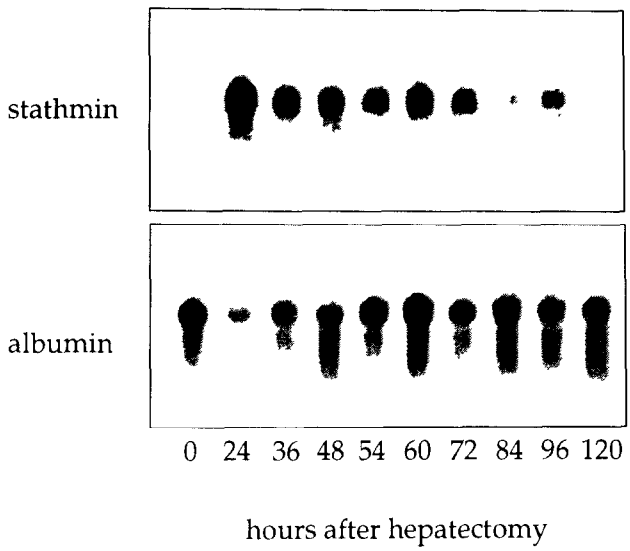

Fig. 5. Northern blot analysis of stathmin mRNA expression after hepatectomy. Stathmin mRNA (1.1 kb) was detected with a ^{32}P-labeled cDNA probe (see section 2) on Northern blots of extracts from rat livers at indicated times after hepatectomy. The same blot was hybridized with a cDNA probe for albumin to reflect variations in total amount of RNA in each lane.

preted as a shift from the quiescent adult to a growing phase comparable to the developmental growth of liver tissue, although with a shorter time course associated with the coordinated growth arrest of hepatocytes during liver regeneration.

3.3. *Phosphorylation of stathmin in developing and regenerating liver*

Stathmin phosphorylation is also known to be regulated in association with physiological and pathological regulation of cell proliferation and differentiation. We therefore examined the distribution of unphosphorylated and phosphorylated forms of stathmin by 2D-PAGE. The overall intensity of the corresponding silver-stained spots confirmed the immunoblotting results, with a significant increase of stathmin at 48 h–72 h after hepatectomy, to a level comparable to that of neonatal liver (Fig. 4).

Since stathmin concentrations are relatively low for silver-stain detection, only its major unphosphorylated form N1 was weakly detected in adult liver and after recovery following hepatectomy. At the highest points of stathmin expression, both around birth and at 72 h after hepatectomy, 3 isoforms were detectable (Fig. 4), the two non-phosphorylated (N1 and N2) and one phosphorylated (P1) form. These results thus further demonstrate the similarity of stathmin implication in developmental and regeneration related liver growth.

Phosphorylation of stathmin could be related in various instances to the regulation of cell proliferation, differentiation and functions (reviewed in [7]). Very recently, it was shown that stathmin undergoes cell cycle-dependent phosphorylation from an unphosphorylated state in S phase to a hyperphosphorylated state in mitosis suggesting that mitotic phosphorylation of stathmin might result in part from direct phosphorylation by cdc2 kinase [41].

Interestingly, no significative change in the phosphorylation of stathmin could be detected during the time course of liver development or regeneration, even at two hour intervals during the first 36 h after hepatectomy, including at the period around 30 h corresponding to the peak of cell divisions and p34cdc2 kinase activity (P. Loyer, D. Glaise, S. Cariou, G. Baffet, L. Meijer, C. Guguen-Guillouzo, unpublished). The relatively low

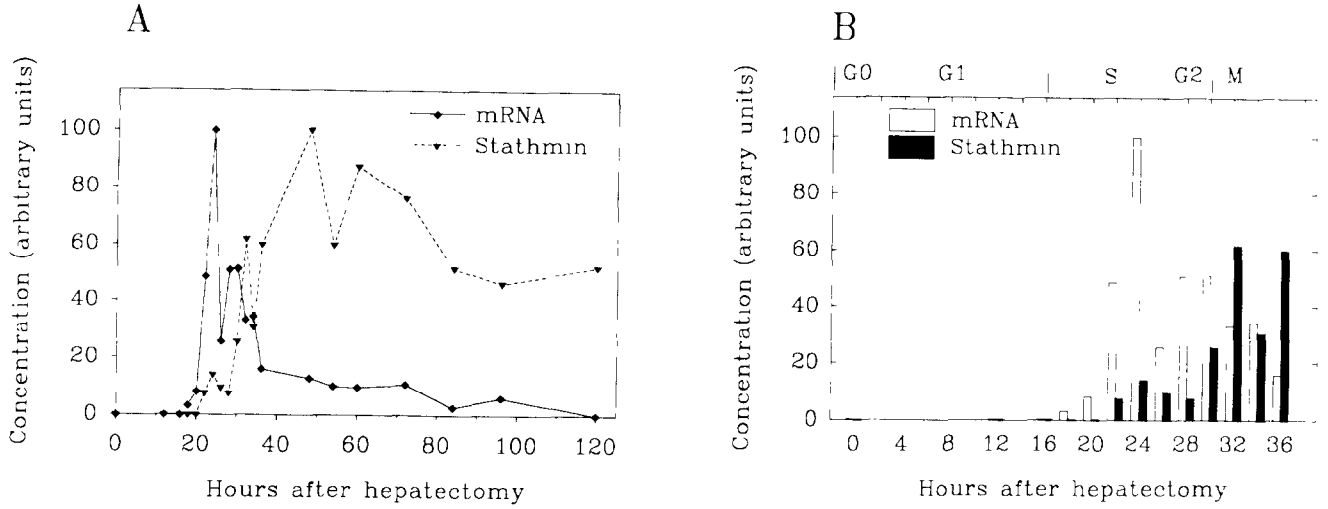

Fig. 6 Compared expression of stathmin mRNA and protein after hepatectomy. Stathmin mRNA and protein were detected and quantified respectively on Northern blots (see Fig. 5) and immunoblots of extracts from the same tissue samples at indicated times after hepatectomy. (A) Five day course of stathmin variation after hepatectomy; (B) close-up on the first synchronised cell cycle after hepatectomy (0–36 h; the various stages of the cell cycle are indicated on top). To normalize for sample variations, stathmin mRNA is expressed as the stathmin/albumin ratio in arbitrary units.

abundance of stathmin in hepatocytes may be partly responsible for the insufficient sensitivity of our phosphorylation analysis. Indeed, this lack of explicit changes of stathmin phosphorylation during liver regeneration could also hide rapid phosphorylation-dephosphorylation events at specific stages of the cell cycle, which may not be detectable in our experimental design due to the relatively low abundance of cells in the corresponding stages, such as for example the comparatively short M phase.

3.4. *Compared expression of stathmin mRNA and protein in regenerating liver*

After hepatectomy, the remaining, regenerating cells enter G1 phase, DNA synthesis starts within 15 h and reaches a peak at 24 h, whereas mitotic activity commences at about 22 h after resection, and mitotic index culminates 10 h later [19]. To evaluate the relevance of stathmin expression in this early period of synchronised cell cycle events, both stathmin mRNA and protein levels were examined in a short-term experiment until 120 h after partial hepatectomy (Figs. 5 and 6). Stathmin mRNA expression was undetectable until 18 h, reached a peak at 24 h and decreased again to a still detectable level between 36 h and 96 h after hepatectomy. In the same liver samples, an increase of immunoreactive stathmin appeared at 22 h and the concentration of the protein augmented until 48 h after hepatectomy (Fig. 6). It is thus interesting to note that although the peak of mRNA expression takes place at 24 h, the highest concentrations of stathmin protein occurs only 24–48 h later, when the mRNA levels have already decreased. This result indicates that stathmin mRNA is probably not very stable, whereas stathmin protein is more stable and accumulates with time even with low amounts of corresponding mRNA.

In various biological systems, regulation of stathmin expression has been related to the regulation of proliferation and differentiation of cells (for review see [7]). Expression of stathmin, a major cytosolic protein in rapidly proliferating lymphocytes, is correlated with periods when cells are cycling through S phase. It was suggested that stathmin is involved in a biochemical pathway leading specifically to down-regulation of DNA synthesis in proliferating peripheral lymphocytes [42,43]. Blocking of stathmin mRNA translation using antisense oligonucleotides delayed entrance of mitotically stimulated lymphocytes into S phase and inhibition of lymphoid proliferation with cyclosporin resulted in decrease of stathmin levels [44]. Our present results show that, although the mRNA for stathmin is maximally expressed during the first mitotic S phase events following hepatectomy, the protein itself is only very partially expressed at this stage and reaches its maximum at least 12 h after the decrease of the mRNA (Fig. 4A,B). However, it is possible that the protein level attained at 24–32 h is biologically relevant and does participate to the regulation of late phases of the first synchronized cell cycle following hepatectomy (Fig. 6B). On the other hand, the larger amount of stathmin accumulated later at its maximum might be rather involved in a more global regulation of cell maturation and termination of proliferation during the course of tissue regeneration, before the decrease of stathmin levels with termination of liver replacement.

A large number of liver mRNA changes were described after hepatectomy [45]. In the 20–40 h interval after resection a peak of mRNA expression was detected for thiostatin, α_2-macroglobulin, β-fibrinogen, α_1-acid glycoprotein, haptoglobulin, and β-tubulin [24]. It is also interesting to note that the ras oncogene product Ha-ras (p21) was increased significantly 36–60 h after partial hepatectomy both at the mRNA and protein levels [25]. Within the same time interval (24–48 h after partial hepatectomy) a 3-fold increase of β-DNA polymerase mRNA was described, paralleled by an increase in enzyme activity [46]. Thus the timing of the main stathmin accumulation was found to roughly coincide with the changes in expression of other proteins involved in regulations of the cell cycle, DNA synthesis and cell proliferation.

Liver regeneration after partial hepatectomy is one of the best in vivo models suitable for the study of cell growth and tissue development. It allows to identify signals which may trigger growth response and make it stop at a fairly predictable point. Using this model we have been able to show that stathmin may participate in the intracellular signalling regulating cell and tissue growth in the liver. This involvement of stathmin in liver growth and regeneration further supports the generality of its proposed role in intracellular regulations as a relay integrating various regulatory pathways of cell proliferation and differentiation during normal development and posttraumatic tissue regeneration. Further studies will be necessary to understand the precise molecular function(s) of stathmin in these regulatory processes.

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