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Cyclin A Is Present in the Endocytic Compartment of Rat Liver Cells and Increases during Liver Regeneration

Marcel Vergés, Anna Castro, Montserrat Jaumot, Oriol Bachs, and Carlos Enrich[1]
Departamento de Biologia Celular, Facultad de Medicina, Universidad de Barcelona, Casanova 143, 08028 Barcelona, Spain

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Recent studies have implicated the cell cycle kinase cdc2 and cyclin A in the inhibition of the fusion of endocytic vesicles *in vitro* during mitosis. However, the presence of cyclins or their associated cyclin dependent kinases (cdks) in the endocytic fractions have not been reported. Using Western-blotting and immunocytochemistry approaches with different anti-cyclin A antibodies we have detected cyclin A in the endocytic compartment of the rat liver. During the pre-replicative phase of liver regeneration the amount of cyclin A in endosomes increases significantly with a peak around 12 hours after partial hepatectomy. Cyclin A-dependent kinases, cdc2 and cdk2, were also found in isolated endosome fractions, showing a distinct kinetics of accumulation during the regenerative period. Finally, histone H1 kinase activity was detected associated with cyclin A in endocytic vesicles and increased in regenerating liver. These results suggest that changes in the organization and in the function of the endocytic compartment during the hepatocellular proliferation may be modulated by proteins involved in the regulation of the cell cycle.

Endocytosis involves the interaction of circulating ligands with their specific receptors located at the plasma membrane and the subsequent internalization of the complex into the cytoplasm through the endocytic compartment. In this compartment is where their pathways diverge, i.e., receptors and ligands are uncoupled and sorted to their appropriate destinations (1,2). In certain conditions, the traffic of receptors and ligands in the liver can be altered. Liver regeneration, induced by partial hepatectomy, is a well characterized *in vivo* model in which resting (G_0) hepatocytes (3) enter G_1 phase of the cell cycle, initiating DNA synthesis with a peak at 24 h after surgery. A subsequent wave of mitosis is accomplished at 28-30 h post-hepatectomy. During regeneration changes in the expression of endosome proteins and in the overall organization of the endocytic compartment have been observed (4,5). A sudden arrest in vesicular traffic at the beginning of mitosis (in both endocytic and exocytic pathways) has been described for different cell types. This phenomenon finally reverts to normal traffic during telophase in coincidence with the recovery of organelle structure (6-10). Cyclins, together with their respective cyclin dependent kinases (cdks), are responsible for the signals that direct the progression of the cell cycle (11,12). Considering that proteins involved in the regulation of the cell cycle are also responsible for the inhibition of endosome fusion during mitosis (13), we have attempted to study the association of cyclins and cdks with endocytic fractions. We report here for the first time that cyclin A is present in the rat liver endocytic compartment. Interestingly, cyclin A increases significantly in the endosomes at the early stages of liver regeneration. In addition, cyclin A was found associated with active cdk2 in the same endocytic vesicles during G_1. Thus, the results suggest that the changes occurring in the endocytic compartment during the hepatocellular proliferation may be modulated by proteins involved in the regulation of the cell cycle.

MATERIAL AND METHODS

Antibodies. The following polyclonal antibodies were used: anti-cyclin A (Santa Cruz Biotechnology, C-22, sc-160), anti-cyclin box (Upstate Biotechnology, 06-179), anti-cdc2 (Upstate Biotechnology, 06-194) and from anti-cdk2 (Upstate Biotechnology, 06-148). Cyclin A control peptide (Santa Cruz Biotechnology, sc-160P) and recombinant cyclin A (fusion protein containing cyclin A from cow, supplied by Dr. G. Warren, Cell Biology Laboratory, Imperial Cancer Research Fund, London, U.K.) were used as controls. In addition, a mouse

[1] Author for correspondence.

Abbreviations: CURL, compartment of uncoupling receptors and ligands; MVB, multivesicular bodies; RRC, receptor recycling compartment.

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monoclonal anti-cyclin A (E43) and a rabbit polyclonal anti cyclin A (JG39) kindly provided by Dr. Tim Hunt and Julian Gannon (ICRF, Clare Hall, UK) were used in some experiments.

Animals. Male Sprague-Dawley rats, weighing 200-250 g, were kept under a controlled lighting schedule with a 12 h dark period. In some experiments, animals were treated with 17-α-ethinyl estradiol (1 mg/ml in propylene glycol) as described to induce the expression of low density lipoprotein receptors (14). Partial hepatectomies were performed according to the procedure of Higgins and Anderson (15), which involves the removal of median and left lateral lobes of the liver (66% of the mass).

Isolation of rat liver subcellular fractions. Endosomes from rat liver were isolated by two different methods, described as Method I (Evans and Flint ref: 16) and Method II, described by Belcher et al. (17) and Jäckle et al. (18).

SDS/polyacrylamide gel electrophoresis and Western blotting. SDS/polyacrylamide gel electrophoresis (PAGE) of proteins was carried out as described by Laemmli (19). In two-dimensional analysis, the slightly modified procedure of O'Farrell et al. (20), described in detail in Enrich et al. (21), was used. Protein content was determined by the method of Bradford (22).

Immunocytochemical methods. Immunofluorescence of frozen semithin (0.5 μm) liver sections was carried out essentially as described (23). In control experiments, the antibody was pre-absorbed with recombinant cyclin A (fusion protein from cow) and also, controls using only the secondary FITC-conjugated antibody were performed.

Immunoprecipitation and histone H1 kinase activity. Endosome fractions were precleared with a normal rabbit serum and protein A-agarose beads. After centrifugation, supernatants were incubated with the anti-cyclin A or anti-cdk2 antibodies for 1 h at 4°C, followed by incubation with protein A-agarose beads under the same conditions. The immunoprecipitates were then washed and the kinase assay carried out in the presence of 3 μg of histone H1 as described by Graña (24).

RESULTS

Rat liver endosomal fractions were isolated from control, sham-operated rats, and from 6, 12, 18, 24, 36 and 48 hours after a partial hepatectomy. As previously demonstrated, the overall protein profiles in endosome fractions isolated from normal and regenerating liver were similar (4,5). Western-blot analysis revealed that the total amount of cyclin A in the homogenates was increased after partial hepatectomy (Fig. 1A). The amount of cyclin A was also elevated in endosomes, showing a peak at 12 h (10-fold) after partial hepatectomy. To rule out the possibility that the stress of the operation or the effect of anesthesia could somehow be involved in this result, endosomes were isolated from sham-operated animals. In this case, no significant changes of the cyclin A were observed throughout the same regenerating period (Fig. 1B).

By Western-blotting, using an anti-cyclin A antibody, a single band of 60 kDa was observed in "early" and "late" endosomes obtained by Method I (see Methods). The amount of cyclin A found in the "late" endosomes being higher than in the "early" endosome fractions (Fig. 2A). The same anti-cyclin A antibody stained the 60 kDa band in highly purified endosome fractions isolated by the procedure refered to as Method II. In this case, cyclin A, was clearly detected in both MVB and CURL endosome fractions and lower amounts were observed in the receptor recycling compartment (RRC) (Fig. 2B).

Two-dimensional analysis by non-equilibrium pH gel electrophoresis (NEPHGE) showed that the 60 kDa polypeptide in endosomes was composed by several spots with a pI in the range between 6 and 7, which is in agreement with the pI observed for cyclin A (Fig.2C).

To get insights into the intracellular localization of cyclin A, frozen sections (semithin, 0.5 μm) from rat liver, fixed in 3% paraformaldehyde, were used for immunostaining with two different rabbit polyclonal anti cyclin A antibodies. Fig. 3, shows the pattern of staining for anti cyclin A (JG39) in normal liver (0), and 12 hours after a partial hepatectomy. The staining was vesicular-like and in some hepatocytes concentrated in the Golgi-lysosomal-endosomal region (23); in regenerating livers of 12 hours the distribution was similar although a large number of structures seem to be labeled specially in the peri-canalicular and the lateral regions of the hepatocytes.

Cyclin A functions as a regulatory subunit associated to Cdk2 or Cdc2 (12). Using antibodies against these cdks, their presence in isolated endosome fractions was demonstrated (Fig. 4.1). Cdk2 and cdc2 showed different temporal expression during the pre-replicative and replicative phases of liver regeneration. In fact, cdc2 could not be detected in the isolated endosome fractions until 24 hours post-hepatectomy (S/G2 phase), after which it increased; in contrast, Cdk2 was present in control liver and showed two peaks at 6 and 24 hours after partial hepatectomy. Finally, to further analyze whether the co-localization of cell cycle proteins in endosomes can result in functionally active complexes, the histone H1 kinase activity was tested after cyclin A (Fig.4.2A), as well as cdk2 (Fig.4.2B), immunoprecipitation. Fig. 4.2A and B, shows that cyclin A and cdk2 associated kinase activity are present in the three endosome fractions, CURL, MVB and RRC, isolated 24 hours after partial hepatectomy. Western-blot analysis of immunoprecipitates using anti-cdk2 antibody, indicated the presence of cdk2 in these immunoprecipitates (Fig.4.2C).

DISCUSSION

It is generally assumed that the main functions for cyclin A and their associated kinases cdk2 and cdc2 take place in the nucleus. However, an increasing number of evidence suggest a role for cyclin A in a variety of non-nuclear functions. Cyclin A has been found in different cellular compartments other than the nucleus: cytosol, plasma membrane and endoplasmic re-

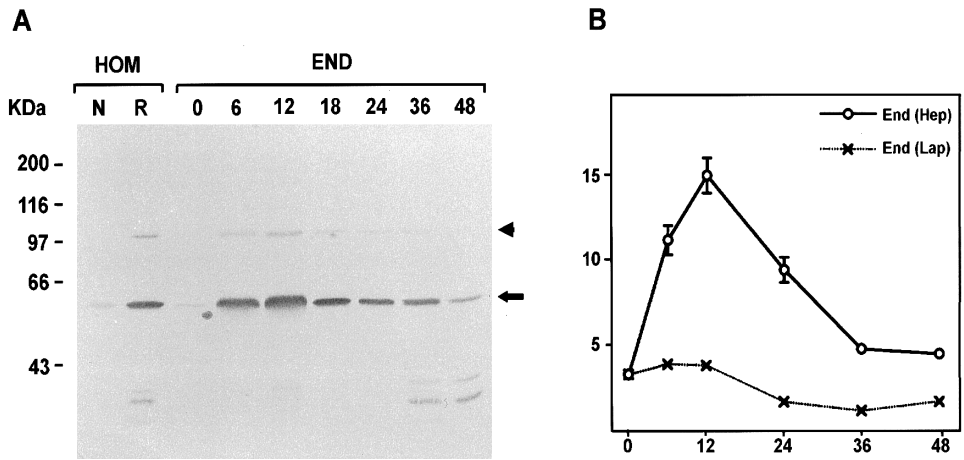

FIG. 1. Expression of cyclin A in endosomes during liver regeneration. (A), shows a representative SDS/PAGE and Western blotting with the anti-cyclin A antibody in endosome fractions (END) and homogenates (HOM) from control (N) and 24 h regenerating livers (R). (B). No significant changes were observed in isolated endosomes from sham-operated animals (laparatomy) at the same points. Area is in arbitrary units. Values are from Western blots of three different experiments and those where standard deviation is not represented varied $<10\%$.

ticulum (28-31). It has been found that the amount of cyclin A in all these compartment increased in proliferating hepatocytes (31). Recently, it has been shown that cdc2, the kinase that controls mitosis entry is responsible for the inhibition of endosome fusion *in vitro* (13) as well as the intra-Golgi mediated transport (25). The inhibition of the fusion of endocytic vesicles seems to be mediated by cyclin A or cyclin B (27) depending on experimental conditions (reviewed by Warren, see reference 10). In spite of these data, the association of cyclin A, cdk2 and cdc2 with endosomes has never been reported. We show here for the first time that, by using western blotting and immunocytochemical analysis, cyclin A is present in the endocytic compartment. We also demonstrate that cyclin A increases in the endosomes of hepatocytes during rat liver regeneration. Cdk2 and cdc2 were also found in isolated endosomes and a concomitant increase of both kinases was detected during liver regeneration although they accumulated with distinct time courses.

Cyclin A/cdk2 complexes were shown to be active in the three endosome fractions when isolated at 24 hours after partial hepatectomy. Since cyclins B1 or E have not been detected in endocytic samples, from rat liver, using specific antibodies (M. Vergés and C. Enrich, unpublished observations), cyclin A is credited to be responsible for the activation of a complex which could be related to membrane fusion processes in which receptor recycling tubules and vesicles are continuously involved.

In the receptor recycling compartment (RRC) the amount of cyclin A was found to be lower than in MVB

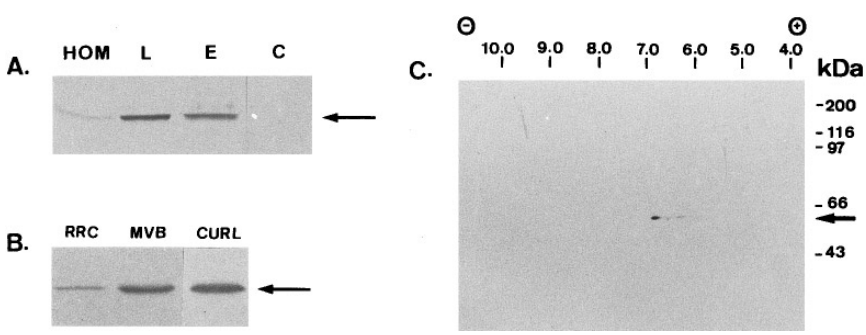

FIG. 2. SDS/PAGE and Western blotting of rat liver endosomes with an anti-cyclin A antibody. (A) Polyacrylamide gel electrophoresis of various endosome fractions, from 24 hours after hepatectomy were analyzed by immunoblotting using an anti-cyclin A antibody. (A) "Late" (L) and "early" (E) endosomes (isolated by Method I) and (C) control, after the depletion of the antibodies with cyclin A peptide; (B), receptor recycling compartment (RRC), compartment of uncoupling of receptor and ligands (CURL) and multivesicular bodies (MVB) (obtained by Method II); (C), two dimensional electrophoresis analysis (NEPHGE), of "MVB" endosomes showed that cyclin A is represented by several spots (arrow) at 60 kDa, pI 7.0-6.0.

and CURL fractions, although on the contrary the histone H1 kinase activity associated to this cyclin in RRC was higher than in the other two endocytic fractions. These results are not in contradiction since the cdk activity is not only dependent on the amount of cyclin A but on the formation of active cdk/cyclin A complexes and the specific phosphorylation of the cdk. Interestingly, the results reported here suggest that cyclin A function in rat liver endosomes could be biphasic: during G_1 it is found associated to cdk2 whereas during G_2/M it could be mostly associated to cdc2. Thus, during G_2/M cyclin A/cdc2 complexes could be involved in blocking the fusion of vesicles. The role of cyclin A/cdk2 during G_1 in endosomes still remains to be elucidated.

The precise role that cyclins and their associated kinases may play in the control of membrane traffic will only be resolved when the targets for the cdks will be identified in the endocytic compartment.

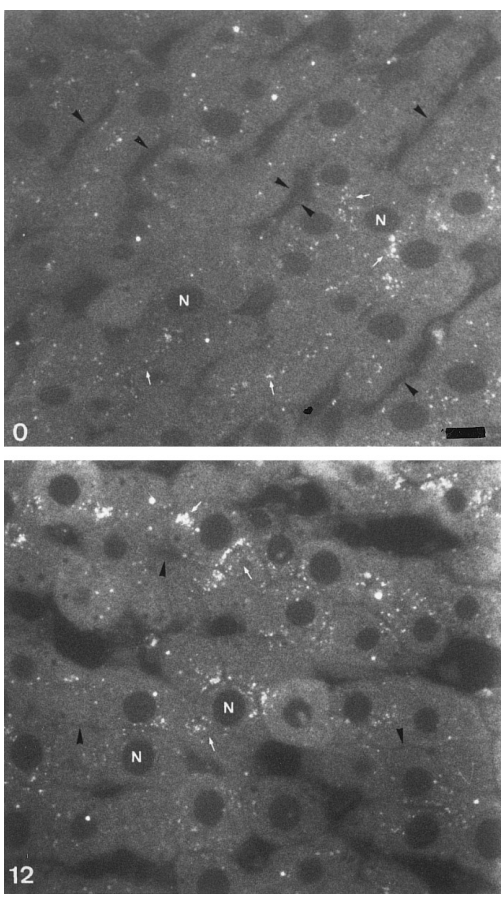

FIG. 3. Immunocytochemical localization of cyclin A in rat liver sections. Rat liver semithin frozen sections (0.5 μm) were stained with a polyclonal to anti-cyclin A antibody (JG-39); 0, control liver and 12 hours after a partial hepatectomy. Small white arrows showed regions of intense staining at the peri-canalicular and lateral regions; arrowheads indicated the position of sinusoids. At 12 hours arrowheads indicated the obliteration of sinusoidal space during the regenerative process. N, nucleus. Bar is 20 μm.

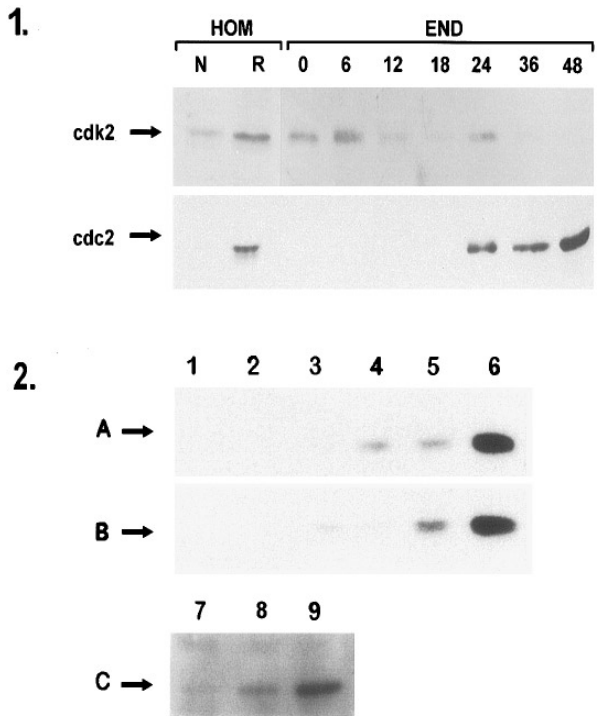

FIG. 4. Association of cyclin dependent kinases to endosomes and immunoprecipitation with anti-cyclin A and anti-cdk2 antibodies and histone H1 kinase reaction. (1) SDS/PAGE and Western blotting with antibodies against Cdk2 and Cdc2 in endosomes (END) and homogenate (HOM) in control (N) and regenerating liver (R, 24 hours). (0), are MVB from normal non-operated liver; (6,12,18,24,36 and 48 hours) endosomes (MVB) isolated at stated hours after partial hepatectomy. (2) Cyclin A (A) and cdk2 (B) were immunoprecipitated in the three endosome fractions isolated (24 hours) by Method II. The histone H1 kinase assay showed a positive reaction in all fractions (4, CURL 5, MVB); 6, RRC). Arrows point to phosphorylated histone H1. (1,2 and 3 are the control experiments, with normal rabbit serum. 7,8 and 9 (C), show the western-blot with the anti-cdk2 antibody of immunoprecipitates from experiment showed in A. In all samples, 50 μg of protein was used.

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