Characterization of Cdk2-Cyclin E Complexes in Plasma Membrane and Endosomes of Liver Parenchyma

INSULIN-DEPENDENT REGULATION

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Rat liver parenchyma Golgi/endosomes fractions harbor a tyrosine-phosphorylated 34-kDa protein. Screening of Golgi, endosomes (ENs), plasmalemma (PM), and cytosolic (Cyt) fractions revealed the presence of the mitotic kinase Cdk2 in ENs, PM, and Cyt. The fluid phase endocytic marker horseradish peroxidase gained access to the endosomal Cdk2, confirming its localization. Cdk2 was shown to be associated to cyclin E and was active in ENs and PM fractions. Treatment administration of a single dose of insulin (1.5 μg/100 g, body weight) induced a time-dependent activation of the insulin receptor kinase in these structures. Insulin receptor kinase activation was followed by the inhibition of immunoprecipitated Cdk2-cyclin E kinase activity in PM and the progressive disappearance of cyclin E. In marked contrast, no such effect was observed in ENs. The injection of a phosphotyrosyl phosphatase inhibitor (bpV-phen) increased the levels of cyclin E in ENs and PM. A massive recruitment of p27kip1 was observed in the Cdk2-cyclin E complexes isolated from PM and Cyt but not from ENs. In vitro, Cdk2-cyclin E complexes have the capacity to inhibit the formation of hybrid structures containing horseradish peroxidase and radioiodinated epidermal growth factor. Therefore, in the PM and ENs of adult rat liver, an active and regulated pool of the mitotic kinase Cdk2-cyclin E and some yet to be defined effectors are present. Cdk2 may contribute to the modulation of transport events and/or maintenance of the topology of endocytic elements.

The endosomal apparatus comprises a complex system of sorting and recycling events, a fact reflected in its fusigenic activity (1, 2). Early studies pointed out that endocytic traffic ceases during mitosis. This suggested that a relationship exists between the components controlling the cell cycle and the endocytic pathway (3), possibly through inhibition of receptor recycling (4), inhibition of the formation of coated vesicles (5), or inhibition of vesicle fusion with continuous budding (6). It was shown that both mitotic extracts and the mitotic kinase Cdk1 associated to cyclins A or B are able to decrease the in vitro fusion of early endosomes (ENs), which supported the idea of an important function in vesicular transport inhibition (7–10). Nuclear checkpoint mechanisms (11–13) are controlled by Cdk5 (14–16). Among the Cdk5s that regulate G1 progression, Cdk2 is activated by association with cyclin E (17) and also dephosphorylation of the Thr-14 and Tyr-15 inhibitory residues located near the ATP binding domain (15, 16). Cyclin binding is required for the kinase cyclin-associated kinase (CAK) to phosphorylate the activating Thr-160 in Cdk2 (18, 19), and inhibitory subunits (cyclin kinase inhibitors) act in part to block cyclin-associated kinase (CAK)-dependent activation of Cdk5 (20). The mechanism of cyclin destruction is a regulated process, and recent results have shown that cyclin E is targeted for proteasome processing through ubiquitination-dependent mechanisms (21–25). Compartmentalization is also an important component in directing the action of Cdk complexes. Hence, it was shown that the N terminus domain is essential for the differential intracellular localization of human cyclins A and B (26), and cyclin B was shown to be localized in the Golgi complex (27).

The process of receptor-tyrosine kinase endocytosis itself was shown to be dependent upon signal motifs contained within the cytosolic domains of the receptors (28–30). The tyrosine kinase tail of the receptors is thought to be required for a maximal rate of endocytosis (34) and tyrosine kinase activity of the epidermal growth factor (EGF) receptor was implicated in both the early (35) and late events of endocytosis (36, 37). A number of putative substrates whose functions are currently under investigation have been identified, suggesting that the action of internalized tyrosine kinases on endocytic transport is made up of several components. These proteins include annexin 1 (38), annexin 2 for the insulin receptor kinase (IRK) (38), pp120 (39), Eps15 (40), EAST (41), and Hrs (42–44). Also, signaling proteins such as phosphatidylinositol 3-kinases are now clearly implicated along the entire trafficking pathway; they are presumed to favor the recruitment of GTPases that function in vesicle budding and fusion (45). During screening for endosomal tyrosine-phosphorylated proteins, we identified a 34-kDa

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1 The abbreviations used are: EN, endosome; G/E, Golgi/endosome fraction; PM, plasma membrane fraction; Cyt, cytosolic; Li and Lh, light intermediate and heavy endosomes, respectively, isolated from the parent light mitochondrial fraction (L); Gi and Gh, Golgi intermediate and heavy endosomes, respectively, isolated from the parent P microsomal fraction; EGF, epidermal growth factor; IKK, insulin receptor kinase; WGL, wheat germ lectin; HRP, horseradish peroxidase; DAB, 3',5'-diaminobenzidine; GST, glutathione S-transferase.

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protein. We show here that an active and insulin-regulated pool of Cdk2-cyclin E complexes is located in hepatic plasma membrane (PM) and ENs.

**EXPERIMENTAL PROCEDURES**

**Reagents, Plasmids, and Antibodies—**Murine receptor grade EGF was purchased from Collaborative Research (Waltham, MA). Insulin was from Sigma. 125I-EGF was prepared using the chloramine-T method (specific activity 100–200 μCi/μg). Rabbit polyclonal monospecific antibodies raised against Cdk1, Cdk2, cyclin E (H12), and cyclin A (C-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal anti-p27kip1 antibody was from Transduction Laboratories (kindly provided by Dr. Claude Labrie, Laval University). Cdk2 complexes were isolated with agarose-conjugated Cdk2 (M2) purchased from Santa Cruz Biotechnology. The compound bpV(phen) (kindly provided by Dr. M. Tremblay (Laval University). Wheat germ lectin (WGL) and protein A-Sepharose were from Amersham Pharmacia Biotech. Histone H1, horseradish peroxidase (HRP; type VI-A), 3′,5′-diaminobenzidine (DAB), and peroxide of hydrogen (H2O2) were from Sigma. [γ-32P]ATP (1000–3000 Ci/mmol) was from NEN Life Science Products. The vector pGEX-2T and GSH-Sepharose beads were obtained from Amersham Pharmacia Biotech. The restriction and modification enzymes (BamHI, EcoRI, NdeI, and T4 DNA ligase) were obtained from Roche Molecular Biochemicals. Isopropyl-β-D-thiogalactopyranoside was from Promega (Madison, WI). The entire intronless DNA sequence of Schizosaccharomyces pombe p13/Suc1 protein subeloned in the expression vector pRK712 (a gift of Dr. Doré through Dr. Sirard, Laval University) was cut with NdeI digested with Klenow polymerase and ligated with EcoRI. The resulting 300-base pair blunt-cohesive fragment was ligated in frame into a pGEX-2T expression vector in which the BamHI site had been digested, filled in, and then digested with EcoRI. Escherichia coli BL21DE3 was transformed and plated, and transformants were selected for ampicillin resistance. Clones expressing the 36-kDa fusion protein GST-p13 were amplified and the fusion protein was linked to glutathione-Sepharose beads according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The T47D cell line (human breast carcinoma mutated for p53) was provided by Dr. Mirault (Laval University). Reagents for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad. All other chemicals were of analytical grade and purchased from Fisher or Roche Molecular Biochemicals. Nylon-supported nitrocellulose filters BA-S 85 were obtained from Schleicher & Schuell (Keils, Québec, Canada). PolyScreen membranes and the Renais-

**RESULTS**

**Cdk2 in Plasma Membrane and Endosomes**

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The supernatant (Cyt) was collected (5 ml/g of liver, 14 ± 2.1 mg of protein/ml, n = 21) and used immediately. For lectin purification of proteins, G/E endosomes were solubilized by agitation at 4 °C for 30 min in the presence of 1% Triton X-100. Solubilized membranes were centrifuged at 100,000 × g for 1 h to remove undissolved materials, and the supernatant was applied to WGL-Sepharose (5 ml packed columns, five cycles at 4 °C). The columns were washed with 40 column volumes of 50 mM Hepes (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM vanadate, 40 mM sodium fluoride, 0.1% Triton X-100 and centrifuged at 50 × g for 2 min. Glycoproteins were eluted by resuspension of each column in 0.3 M N-acetyl-D-glucosamine for 60 min at 4 °C. The columns were spun to yield the eluates (112 ± 22 µg/ml). The eluate contents from any of the fractions were then used for the modification of the Bradford’s method using bovine serum albumin as a standard.

**Kinase Assays—**Cdk2 activity was assessed in vitro by histone H1 phosphorylation as described previously (49) and modified as follows. Freshly isolated cytosol or solubilized membranes (0.5 mg of cell fraction protein) were immediately incubated in the presence of the Cdk2 antibody (1 µg/ml), 125I-EGF (100 ng/ml) (kindly provided by Dr. M. Tremblay (Laval University)). They were injected with insulin via the jugular vein at the concentration of 5 and 0.2 mM, respectively, along with a trinucleotide-regenerating system (54). The reaction continued for 20 min at 25 °C in a water bath and was stopped by applying a 50-µl aliquot onto a piece of Whatman P81 paper (2.5 cm2). The paper squares were washed three times with water and air-dried, and radioactivity was measured by scintillation counting. The phosphorylation of ENs in vitro and KOH treatment of the gels was done as previously reported (50, 51). Statistical analysis was done with Statview (Abacus Concepts Inc., Berkeley, CA).

**Fusion Assay—**The fusion reaction and the density shift procedures were adapted from Woodman and Warren (52) and Courey et al. (53), respectively. ENs (G/E containing HRP or 125I-EGF were isolated, pelleted, and immediately resuspended (1 mg of protein/ml) in the sucrose-imidazole buffer (250 mM sucrose, 2 mM MgCl2, 50 mM KCl, 1 mM sodium azide, 3 mM imidazole, pH 7.0). The two populations of ENs (0.5 mg of protein cell fraction each) were combined with 0.5 ml of 3.85% of protein) of diluted (1:1) Cdk2-depleted cytosol, and the volume was adjusted to 3.70 ml with sucrose-imidazole buffer. 200 µl of active Cdk2 isolated on GST/p13 suc1 affinity chromatography the same day from solubilized rat liver ENs (5 µg of protein per milliliter) was added. In the control reaction, 200 µl of cytosol buffer was added (25 µl GSH in phosphate-buffered saline, pH 7.4). The fusion reaction was started at 37 °C by adding 100 µl of a mixture containing ATP and GTP to a final concentration of 5 and 0.2 mM, respectively, along with a trinucleotide-regenerating system (54). The reaction continued for 15 min and was stopped by adding EDTA to a final concentration of 10 mM and 0.5 ml of DAB solution (55 mM DAB, 0.74 mM sucrose, 3 mM imidazole, pH 7.0) and 32 µl of 6% H2O2 in 0.25 mM sucrose-imidazole buffer. The incubation continued for 30 min with gentle agitation. The HRP-containing ENs were then separated from the other elements by centrifugation over a density gradient for 3.25 h at 100,000 × g in a Beckman SW 28 rotor. Fractions (0.5 ml) were collected from the bottom of each tube, and the refraction index was measured to determine density (refractometer from Fisher). Aliquots of each fraction were assessed for 125I content using a γ-counter (Wallac, Turku, Finland). Shifted endosomes containing 125I represented 18–26% (8000–26,000 cpm; n = 6) of the total pool of 125I-containing ENs.
proteins, mainly proteases or protease inhibitors, were also identified (Fig. 1A). Based on its apparent molecular weight and previous putative function in vesicular fusion (8), we hypothesized that the 34-kDa protein was a mitotic kinase. The incubations in the presence of GST-p13 suc1-Sepharose beads resulted in 30% depletion of the 34-kDa bands (Fig. 1B). This can be explained in part by the presence of a fragment of a protein related to VASAP-60 (56). The incubations in the presence of [γ-32P]ATP confirmed the presence of a 32–34-kDa protein associated with GST-p13 suc1 beads. Three phosphorylated isoforms were observed, including a more slowly migrating alkali-resistant one, characteristic of Tyr-15 phosphorylation (Fig. 1C). Since the appropriate temporal activation of cyclin-E Cdk2 and cyclin A-Cdk2 is required for the G1- to S- and S phase progressions, respectively (15, 16), we then examined the presence of the activators cyclin E and cyclin A by using monospecific antibodies. Cyclin E, but not cyclin A, was recovered in the endosomal glycoprotein fraction (Fig. 1D). Since there was minimal contamination (less than 1%) by cytosolic proteins in our fractions, these results made credible the presence of a mitotic kinase and its activator cyclin E in endosomes and/or Golgi elements and also showed that cyclin E and a mitotic kinase, presumably Cdk2, have the capacity to associate directly or indirectly with glycoproteins. The presence of Cdk2 in the G/E, PM, and cytosolic fractions but not in a nuclear fraction (not shown) was confirmed by either direct immunoblotting or affinity precipitation using GST-p13 beads (Fig. 2). Approximately 75% of the total Cdk2 was located in the cytosol, 15% in the PM, and 5% in the G/E fractions (Fig. 2B). The G/E fraction used in this study was shown to contain Golgi elements but to be essentially free of plasmalemma and other subcellular constituents (47, 48, 57). This was affirmed here by the detection of the slower migrating Cdk2 isoform in the cytosolic fraction, which was barely detected in the G/E and PM fractions (Fig. 2A). The faster migrating isoform detected in the PM and G/E fractions was characteristic of phosphorylation at the activating Thr-287 (18). We concluded then that a relatively small but consistent and presumably active pool of Cdk2 was associated with membranes. To refine the compartmentalization of Cdk2, we purified L ENs originating from the parent light mitochondrial fraction and G ENs originating from the parent P Golgi fraction (47). We found that Cdk2 was enriched in Li ENs, a highly homogeneous fraction containing late ENs and a negligible amount of the marker enzymes sialyltransferase and galactosyltransferase. A 50% less intense signal was systematically detected in the Gi fraction representing early ENs containing Thr-267 (18). We investigated whether fluid phase endocytosed HRP could gain access to Cdk2 to verify its endosomal localization. This experiment was based on the fact that intraluminal polymerization of the HRP substrate DAB induces loss of antigenicity partly due to the elimination of detergent-soluble proteins (58). Intravenous injection of HRP, isolation of G/E fractions, and subsequent polymerization of DAB in vitro was followed by a time-dependent

Fig. 1. Phosphorylation of endosomes in vitro. A, endosomes (G/E, 5-mg proteins) were subjected to phosphorylation with 1 mM ATP at 37 °C for 15 min as described under Experimental Procedures. They were solubilized and glycoproteins were purified using WGL-Sepharose affinity chromatography and then immunoblotted (25 μg of proteins) using anti-phosphotyrosine antibodies. B, endosomes were phosphorylated, and glycoproteins were purified as above. The glycoproteins (0.2 mg of proteins) were incubated for 60 min at 4 °C in the presence of 50 μl of GST-p13 suc1-Sepharose beads (+) or Sepharose beads (−). The eluted proteins were separated by SDS-polyacrylamide gel electrophoresis and then immunoblotted using anti-phosphotyrosine antibodies. C, endosomes (G/E, 5 mg of proteins) were subjected to phosphorylation with 1 mM [γ-32P]ATP (specific activity, 12 μCi/nmol) at 37 °C for 15 min. Membranes were then solubilized, and glycoproteins were purified using WGL-Sepharose affinity chromatography. The glycoproteins were subjected to GST-13 suc1-Sepharose affinity chromatography, and the eluted 32P-labeled proteins were separated by SDS-polyacrylamide gel electrophoresis, alkali-treated, and autoradiographed. Left, autoradiography of total labeling; right, the same gel was submitted to KOH hydrolysis. D, endosomal glycoproteins purified by WGL- Sepharose affinity chromatography were immunoblotted (25 μg of proteins) using anti-cyclin E (lane 1) or anti-cyclin A (lane 2) antibody.

Fig. 2. Localization of Cdk2 in endosomes, plasma membrane, and cytosolic fractions of rat liver. Cell fractions were isolated and submitted to immunoblotting analysis directly using the anti-CDK2 antibody or after a prior precipitation using GST-p13 suc1-Sepharose beads. A, immunoblots of cell fractions (100 μg of proteins). Lane C, control whole extract of dividing T47D-H3 cells. B, the signals were submitted to densitometric analysis, and the results were expressed as a percentage of the total cellular Cdk2. Each value represents the mean ± S.D. of three independent experiments. C, immunoblot of Cdk2 precipitated with GST-p13 suc1-Sepharose beads (0.5 mg of proteins).
loss of signal for Cdk2 in the detergent-soluble fraction. In contrast, the signal for Cdk2 was not affected in the PM fraction 15 min after the injection of HRP (Fig. 3A). To determine the nature of the association of Cdk2 with membranes, ENs (G/E) were treated with high salts and carbonate, pH 10 (51). The results indicated that Cdk2 was firmly associated to ENs. The signal for Cdk2 was lost, however, following the treatment of permeabilized or nonpermeabilized ENs with a protease, favoring the idea that Cdk2 is cytosol-oriented (Fig. 3B).

Kinase Activity in Hepatic Fractions—To estimate the relative activity of compartmentalized Cdk2, equal amounts of proteins were immunoprecipitated from each fraction by using the anti-Cdk2 antibody. The immunoprecipitates were then subjected to a kinase assay using histone H1 as a substrate. Based on equal amounts of proteins, the kinase activity of cytosolic Cdk2 represented less than 5% of the endosomal activity (Fig. 4A). We confirmed that the Cdk2 activator cyclin E (but no cyclin A) was detected in the hepatic fractions by direct immunoblotting using a monospecific antibody. The 52-kDa cyclin E was less abundant in the cytosol, explaining in part the apparent low activation state of Cdk2. This was most probably due to a rapid degradation (22), also suggested by the presence of a number of fragments. Of note, a similar profile was not observed in the PM and the G/E fractions (Fig. 4B). In addition, the Cdk2 inhibitor p27kip1 (18) was also easily detected in the cytosolic compartment and barely detected in PM or ENs (Fig. 4C). To confirm the formation of active complexes, Cdk2 was directly precipitated prior to immunoblotting. In these conditions, the results showed the presence of cyclin E/p27kip1/Cdk2 complexes (Fig. 4D). Taken together, these results confirmed that Cdk2 and its regulators are present on the cell surface and endocytic membranes of the adult rat liver and have the capacity to form active complexes.

Regulation of Cdk2-Cyclin E Complexes—Cdk2 is a serine/threonine kinase that integrates a number of positives and negatives signals through phosphorylation/dephosphorylation and protein-protein interactions (20). While Cdk2 was readily phosphorylated in vitro (Fig. 1B), preliminary experiments performed in both PM and ENs did not indicate consistent changes in the phosphorylation status of Cdk2 once stimulated by EGF or insulin. This was verified by the electrophoretic migration profiles of Cdk2 and by immunoblots of Cdk2 precipitates using the anti-phosphotyrosine antibodies (not shown). However, 30 min following the injection of a subsaturating dose of insulin (1.5 µg/100 g, body weight), the Cdk2 kinase activity was abolished in the PM fraction. In contrast, no such effect on Cdk2 kinase activity by insulin was observed in ENs (Fig. 5A). IRK is a transmembrane tyrosine kinase that is rapidly delivered to hepatic ENs with maximal accumulation occurring 2 min after injection of insulin (46). We confirmed the time course of internalization of IRK following the injection of insulin. IRK was rapidly tyrosine-phosphorylated and then disappeared progressively from the cell surface to reappear in ENs (Fig. 5B). Under these circumstances, cyclin E levels reached more than 2-fold the basal value 2 min following the injection of insulin (p < 0.001) and then decreased as the kinase-activated receptor disappeared from the cell surface (Fig. 5B). This effect was also investigated in ENs at the time peak of concentration of IRK (2 min) and a later time (15 min) that corresponded to a 50% decrease in IRK endosomal content for this dose (46). No effect on cyclin E was observed in ENs (Fig. 5C).

To verify the involvement of tyrosine phosphorylation events, we injected a potent PTP inhibitor bpV(phen) (59). Cyclin E levels were markedly increased both in ENs and PM but not in the cytosolic fraction (Fig. 6C). Interestingly, bpV(phen)-dependent recruitment of p27kip1 was observed in both the Cdk2 (Fig. 6A) and cyclin E (Fig. 6B) complexes isolated from the PM and Cyts but not the ENs fraction. Under these conditions, the immunoprecipitated Cdk2 appeared also tyrosine-hyperphosphorylated as assessed by the shift in electrophoretic mobility both in the PM and cytosolic fractions (Fig. 6).

Effect of Exogenously Added Cdk2 on Fusion Events in Vitro—To relate the compartmentalization of active Cdk2 with a functional consequence, we investigated the capacity of exogenously added Cdk2, purified from the G/E fraction by using Sepharose-GST-p13affinity chromatography, to inhibit the formation of hybrid ENs in vitro. In liver parenchyma, EGF was previously shown to be internalized along with its receptor in a time- and dose-dependent manner with the maximal concentration being attained 15 min after injection of the ligand (48, 60, 61). In contrast to insulin, which was found to rapidly dissociate and degrade, EGF remains bound to its receptor in hepatic ENs (62). We therefore tried a modified assay where two populations of ENs containing either the fluid phase internalized HRP or 125I-EGF endocytosed at an early time (5 min) were mixed in the presence of a cell-free fusion system (52). The density of HRP-containing ENs was then modified by the polymerization of DAB, and these were centrifuged in a density gradient (53). The results showed a peak of hybrid bodies at a mean density of 1.203. The amount of hybrids was decreased by 80% when Cdk2 was added during the fusion reaction (Fig. 7).

DISCUSSION

In this report, we show that an active pool of Cdk2 is associated to PM and ENs of liver parenchyma cells. The fractions
FIG. 4. Cdk2 activity in plasma membrane, endosomal, and cytosolic fractions and presence of Cdk2-cyclin E-p27 complexes. A, the Cyt, G/E, and PM Cdk2 was precipitated (0.5 mg of starting proteins) and submitted to a kinase assay using histone H1 as a substrate. The activities were expressed per 25 μg of protein cell fraction, and results were expressed as percentage of the maximal value. Mean ± S.D. of three experiments. B, fractions (100 μg of proteins) were submitted to immunoblotting analysis using an antibody against cyclin E. C, the same membrane was revealed with the anti-p27kip1 antibody after stripping of the signal for cyclin E. D, solubilized G/E and PM fractions (1 mg of proteins) were immunoprecipitated using Cdk2-agarose beads. The precipitated proteins were submitted to immunoblot analysis using anti-cyclin E and anti-p27kip1 antibodies. Pieces of the same membrane were incubated separately with the indicated antibody.

FIG. 5. Time course of changes in Cdk2 activity, insulin receptor-kinase activation, and cyclin E content in plasma membrane and endosomes following the injection of insulin. A, PM and G/E EN were prepared at the noted time following the injection of a sub saturating dose of insulin (1.5 μg/100 g, body weight). The fractions were solubilized (1 mg of protein), Cdk2 was immunoprecipitated using the anti-Cdk2 antibody, and the immunoprecipitated proteins were submitted to the kinase assay using histone H1 as a substrate as described under “Experimental Procedures.” Values are mean ± S.D. (n = 3). *, statistically different from control (time 0), Student’s t test (p < 0.001). B, PM fractions were prepared at the indicated time following the injection of insulin (1.5 μg/100 g, body weight). Solubilized proteins (100 μg) were submitted to immunoblot analysis using p-Tyr 02, a960, anti-Cdk2, and anti-cyclin E antibodies. The signals for cyclin E were subjected to densitometric analysis and expressed as a percentage of the maximal value (p < 0.005; 0 min versus 2 min, n = 3, Student’s t test). C, G/E fractions were prepared at 2 min (time peak of IR internalization) or 15 min following the injection of insulin (1.5 μg/100 g, body weight) and then immunoblotted (100 μg of protein) using a960, p-Tyr 02, anti-Cdk2, or anti-cyclin E antibodies. The signals for cyclin E were submitted to densitometric analysis and expressed as a percentage of the maximal value. Results are the mean ± S.D. of three independent experiments.
Intravenously with vehicle or bpV(phen) (1 mmol/L), and cytosol of bpV(phen)-treated rats. Tethering cyclin A-Cdk2 complexes by p21-PCNA complex to membranes is unclear at this point; however, a glycoprotein seems to play an important role (Fig. 1).

The mechanism of targeting of the Cdk2 inhibitor p27kip1 against Cdk2, cyclin E, and p27kip1 solubilized fractions (100 μg of starting proteins). Pieces of the same membrane were submitted to analysis with monoclonal antibodies against Cdk2, cyclin E, and p27kip1. Used in this study were characterized by enzyme markers, silver staining, electron microscopy, and receptor-mediated endocytosis (46–48, 57). A minimal contamination by cytosolic complexes. The fact that no p27kip1 recruitment was apparent in ENs confirmed that the Cdk2 activity is not affected by these regulatory processes at this locus. A simple hypothesis would be that the endosomal Cdk2 is located in the luminal side of endocytic elements. However, this was not supported by the oriented proteolysis experiments (Fig. 3B).

Alternatively, the degradation of the complexes may be handled by other mechanisms; in this regard, we have identified a number of tyrosine-phosphorylated protease inhibitors by fingerprinting/mass spectrometry coupled to data base identification.

There is a potential important consequence for the presence of active Cdk2 associated with PM and ENs. It was reported that both mitotic extracts and Cdk1 associated with cyclin A or B have the ability to inhibit vesicular fusion events in vitro (8–10). This was confirmed here for the kinase Cdk2-cyclin E (Fig. 7). Mitotic kinases are also thought to play an important role in the disruption of the Golgi complex and subsequent redistribution of Golgi-derived vesicles in daughter cells during mitosis (6, 65). A marked increase in the amount of cyclin A during liver regeneration also supported the idea that Cdks are involved in the arrest of vesicular traffic during mitosis (66). We were unable to detect either Cdk1 or cyclin A in our fraction. However, the fact that (i) Cdks have a relatively large spectra of phosphorylation in vitro (67), (ii) an active membrane-bound Cdk2 was present in PM and ENs, and (iii) this Cdk could inhibit the formation of hybrid organelles in a cell free system lends firm support to the view that compartmentalized Cdk2-cyclin E complexes play an important role in the vesicular fusion reaction in vivo.

Functions for Cdk2-cyclin E as well can be anticipated in endocytic transport. For example, Cdk2-cyclin E might restrain receptors endocytosis. Also, by inhibiting the fusion process, Cdk2-cyclin E may help to avoid unwanted homotypic or heterotypic fusion events. In this case, its role would be reminiscent of checkpoints controlling the quality of the DNA replication process during progress into the mitotic cell cycle (14–16). Of note, the intracellular traffic of an EGFR-green fluorescent protein chimera was recently followed in real time. A flickering behavior of varicocities along endosomal tubules and their subsequent fusion with more stationary compartments was consistent with the occurrence of biochemical mechanisms that tightly control the timing of the endosomal fusion (68). In addition, the Cdk2-cyclin E complexes may
also function in controlling the topology of more stationary compartments (early endosomes, multivesicular bodies, late endosomes) by inhibiting fusion while budding continues (6). Such an important function is consistent with a more constitutive activity of Cdk2 complexes in ENs.

The precise molecular mechanism underlying the action of cyclin E-Cdk2 should be resolved by identifying the targets in the endocytic and cell surface compartments involved either in the dynamics of microtubules (69, 70) or the fusion process (71). In this regard, quiescent hepatocytes in vivo exhibit cyclin D1 and cyclin E levels similar to those observed during liver regeneration (72). This nonmitotic Cdk2-cyclin E activity appeared to be directed against 78- and 105-kDa substrates in differentiated osteoblasts (73). In conclusion, an active and ligand-regulated pool of Cdk2-cyclin E is tightly associated to PM and ENs and has the ability to inhibit vesicular fusion events. Compartmentalized Cdk2-cyclin E may be an important component sensing endocytic transport and/or the topology of the endosomal apparatus.

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Characterization of Cdk2-Cyclin E Complexes in Plasma Membrane and Endosomes of Liver Parenchyma: INSULIN-DEPENDENT REGULATION
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