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Enhanced Prenyltransferase Activity and Rab Content in Rat Liver Regeneration

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Rabs are small GTP-binding proteins with a regulatory role in intracellular vesicular traffic. The modulation of their levels and activity in different physiological situations is poorly understood. During the first cell cycle of rat liver regeneration we observed a differential regulation of some Rabs, with a progressive increase of those involved in exocytosis and a progressive decrease of one involved in endocytosis. This could be related with the need of exposing growth factor receptors and prolonging the transduction of their signal in preparation for mitosis. Moreover, we observed an increased activity of protein prenyltransferases, the enzymes responsible for the prenylation of several proteins involved in crucial processes of proliferation, without a corresponding increase in the amount of prenyltransferase protein.

Key Words: **intracellular traffic; Rab proteins; cell proliferation; regenerating liver; prenyltransferases; prenylation.**

The hepatocyte is a highly polarized cell, where active vesicular traffic occurs. Examples of its trafficking complexity include the endocytic uptake of many macromolecules such as asialoglycoprotein, transferrin and LDL (low density lipoprotein) receptors from the sinusoidal blood, the transcytotic movement of molecules such as IgA receptor, and the exocytic insertion of transport proteins into the canalicular membrane crucial for bile formation (1). During liver regeneration that follows partial hepatectomy in the rat, hepatic metabolism increases, so vesicular traffic is also likely to be enhanced. Moreover, during this proliferative process, the levels of several membrane receptors are changed (2, 3). Some receptors decrease without changes in biosynthesis (4–6), implying an altered balance between exposed receptors and intracellularly stored receptors. These data suggest that in the regenerating liver, intracellular vesicular traffic is regulated differently.

Several proteins have been proposed as regulators of vesicular traffic. Prominent among these, are the Rabs, small monomeric GTPases, that bear one or two geranylgeranyl groups at their C-termini. Rabs function in the processes by which membrane vesicles identify and fuse with their target vesicle (7). For example, each transport step of the endocytic and secretory pathways bears a distinct set of Rabs. Therefore, each step of the intracellular traffic seems to be independently regulated by a specific Rab protein (8). Rabs bound to GTP, enter the membranes of the donor compartment and are active in targeting specific vesicles; after targeting, the GTP/GDP exchange occurs, which allows the Rabs to return to the cytosol in an inactive form.

The subcellular distribution of the Rabs between membrane and cytosol is considered to be indicative of their active state (8). The membrane association of some Rabs can be modulated by phosphorylation with cell-cycle-specific kinases during mitosis (9), and by growth factor-activated kinases during interphase (10).

Little is known about the regulation of the levels of Rabs. A few studies have demonstrated a higher level of some Rabs in cells particularly engaged in vesicular traffic (11, 12). The study of these small prenylated proteins is particularly interesting in the regenerating liver, since increased protein prenylation and increased GTP binding to proteins in the 20–30 kDa range (13) have been observed in this system.

Prenylation is a posttranslational modification that increases the hydrophobicity of the proteins and consequently their capacity to associate with membranes (14). Prenylation is catalyzed by specific cytosolic enzymes, the protein prenyltransferases, that covalently attach the farnesyl group (farnesyl transferase, FTase) or geranylgeranyl group (geranylgeranyl transferase,

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GGTase I and II) to cysteine residues in specific sequences at the protein carboxy-terminus (15). Among the farnesylated proteins are nuclear lamins and Ras, whereas geranylgeranylation of proteins such as Rho and Rac is catalyzed by GGTase I and that of Rab proteins by GGTase II (Rab prenyltransferase).

Little is known about prenyltransferase regulation. In 3T3-L1 adipocytes, insulin has been shown to phosphorylate the alpha subunit common to both FTase and GGTase I and activate these enzymes (16, 17). Nothing is known about prenyltransferases in regenerating liver. However, several prenylated proteins are known to be changed (18, 19).Therefore we have studied the amount and activities of the prenyltransferases and the levels of Rab proteins at different times during the first cell cycle after partial hepatectomy. The chosen times, 4 h, 16 h and 24 h, correspond to G l phase, onset of S phase and onset of mitosis, respectively. Several Rab proteins responsible of specific trafficking pathways were considered: Rab 2 and Rab 8 are involved in the exocytic-secretory pathway; Rab 5 is involved in the first stages of endocytosis; Rab 4 is involved in the recycling of endocytic vesicles to the plasma membrane. Their subcellular distribution was also determined, in order to assess their state of activation.

MATERIALS AND METHODS

Animals. Sprague–Dawley male rats (180–200 g bw), fed with standard diet *ad libitum*, were kept in a controlled dark–light cycle (daylight from 6 AM to 6 PM). Partial hepatectomy (P.H.) was performed as described by Higgins and Anderson (20) under light ether anaesthesia. The rats were sacrificed 4, 16 and 24 h after the operation. Not operated (N.O.) rats were utilized as controls after establishing that sham operated rats behaved in the same way. The livers were stored at −70°C until use. All animals received human care.

Particulate and soluble fractions. Liver was homogenized in 5 volumes of 25 mM Tris HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM PMSF and 0.1 μM leupeptin. The 1000-g post-nuclear supernatant was centrifuged at 100,000*g* for 2 h, in order to obtain a membrane pellet (particulate fraction) and a cytosol supernatant (soluble fraction).

Immunoblotting. Immunoblotting of the prenyltransferases (FTase, GGtase I and II) was performed on cytosolic proteins separated by 10% SDS–PAGE and transferred to nitrocellulose paper. FTase and GGTase I were revealed using primary rabbit antibodies (1:2000) against the respective beta-subunits. GGTase I antibody raised against a maltose binding protein fusion with the GGTase I β-subunit and FTase antibody raised against a GST fusion to FTase-bovine β-subunit were the generous gift of Charles A. Omer, Merck Research Laboratories. GGTase II was revealed, using a primary antibody (1:2000) raised (Cocalico Biologicals) in the rabbit against the synthetic peptide Cys-AspVal-Thr-lle-Lys-Ser-Asp-Ala-Pro-Asp (the N-terminal sequence of rat GGTase Il β-subunit) linked through the Cys to KLH.

Immunoblotting of Rab proteins was performed on both the particulate and the soluble fractions, resolved by 12.5% SDS–PAGE and transferred to nitrocellulose paper. Rabs were revealed with specific rabbit polyclonal antibodies (Santa Cruz Biotechnology) (1:100).

The bound antibodies were visualized by the alkaline phosphatase reaction, using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine (Sigma) as substrate. Scanning densitometry of the filters quantitated the amount of protein.

Prenyltransferase activity. FTase and GGTase I activities were assayed utilizing biotinylated peptides as prenyl acceptors, by adaptation of a previously described method (21). Assays contained, in a final volume of 40 μl, the following components: 0.5 μM ^{3}H-farnesylpyrophosphate (FPP) (10–20 Ci/mmol) or 2.1 μM ^{3}H-geranylgeranylpyrophosphate (GGPP) (15–30 Ci/mmol), liver cytosol (6–10 μg protein), 50 mM phosphate buffer, pH 7.0, 4 mM $MgCl_2$, 40 μM $ZnCl_2$, 10 mM DTT, and 30 μM Bt-KTKCVIS or Bt-KKFFCAIL, for FTase or GGtase I, respectively. After incubation for 30 min at 37°C, the prenylated biotinylated peptide-product was bound to avidin-agarose beads, washed and collected on Gelman GN-6 membrane filters (0.45 μM mesh) with an Amicon Multivacuum filtration unit. The filters were solubilized in Hyonic Fluor (Packard) scintillation fluid and analyzed for radioactivity.

Other methods. Protein concentration was determined by the method of Lowry (22) using bovine serum albumin as a standard. Scanning densitometry using a NIH Image 1.51 program with a Macintosh computer quantitated all the images.

RESULTS

The levels of prenyltransferases were determined by Western analysis on cytosol liver proteins at various times after partial hepatectomy (Fig. 1).

FTase protein showed a progressive decrease during regeneration, with levels reaching 50% of the control after 24 h. GGTase I also showed a decrease in mass during the first cell cycle, although it was less evident than with FTase. GGTase II, that is specific for the prenylation of Rabs, showed no significant change from the control until 16 h, but exhibited a 30% decrease at 24 h.

Since a general decrease in the mass of the three enzymes was observed, any increased prenylation activity observed during liver regeneration cannot be explained by changing enzyme protein levels. The enzymatic activities of FTase and GGTase I were determined for comparison (Fig. 2). The GGTase II activity was not evaluated because the Rab substrates were not available. The results show that activities of both FTase and GGTase I underwent a net progressive increase during the first cell cycle. Ftase activity was double that of the control at 16 h, and three times the control at 24 h. GGTase I activity was already increased at 4 h, and even higher at the following times. The data suggest that the demand for prenylation is met by increased activity of the enzymes, rather than an increased mass.

The levels of Rab proteins, which are particularly significant in the function of the exocytic and endocytic pathways, were measured at the same times of regeneration. Since the Rabs are found in the particulate and cytosolic fraction, which correspond to the membrane associated (GTP form) and the soluble (GDP form) proteins, respectively, evaluation of the Rabs'

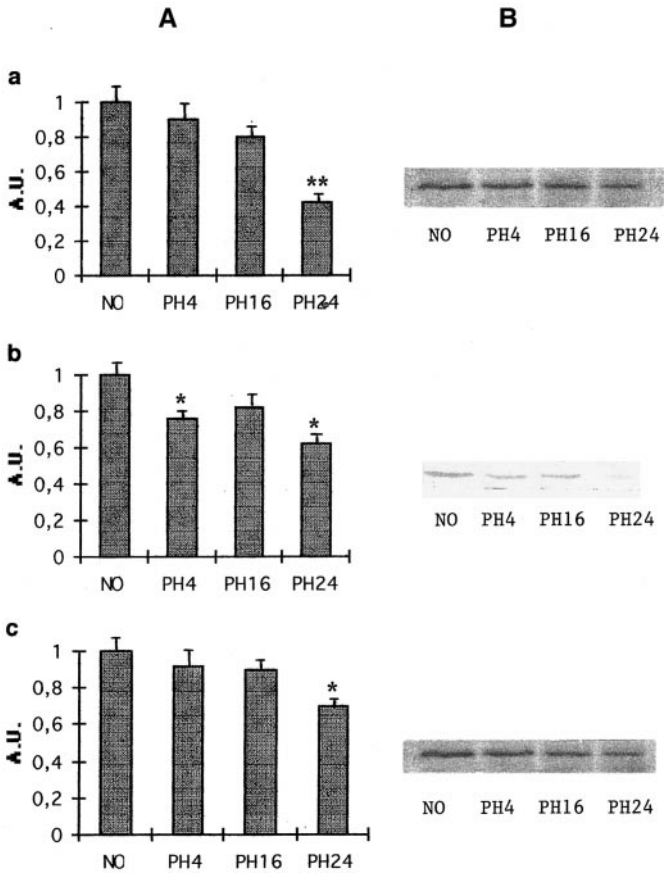

FIG. 1. Levels of prenyltransferases FTase (a), GGTase I (b) and GGTase II (c) in liver cytosol of not operated (NO) or partially hepatectomized animals from 4 (PH4), 16 (PH16), and 24 (PH24) h. (A) Densitometric scanning: absorbance is expressed in arbitrary units (A.U.) with respect to NO set to 1 (mean + S.D., $n = 3$ exp.). $^{*}P < 0.01$; $^{**}P < 0.001$. (B) Representative immunoblot. Each lane contained 150 μg proteins.

distribution would reflect the active state of the proteins, membrane associated Rabs being active and soluble Rabs being inactive.

Rab 2, that is localized in the ER-Golgi area, functions in the early stages of the secretory pathway. Its levels were significantly increased in the particulate fraction after partial hepatectomy, reaching a level double than the control at 4 and 24 h. In contrast, Rab 2 in the soluble fraction increased only at 24 h (Fig. 3a). These observations suggest a more active state at earlier times and an increased synthesis later.

Rab 8, that is involved in the transport from the *trans*-Golgi network to the basolateral membrane, increased both in the particulate and the soluble fractions (Fig. 3b).

Rab 4, that is responsible of the recycling pathway, progressively increased in the membrane fraction, and slowly decreased in the cytosol (Fig. 3c). These results suggest both a redistribution to the membrane and an increased biosynthesis.

Rab 5, the marker of the endocytic pathway, behaved differently from the other Rabs examined. It decreased both in particulate and soluble fractions, with a minimum at 4 h (Fig. 3d).

These data show that partial hepatectomy affects all the Rab proteins examined, but with different timing and in different ways. Rab 4 in particular showed the most significant subcellular redistribution.

DISCUSSION

Prenylated proteins are involved in several central processes of cell metabolism, and prenylation is essential for their functionality. Therefore the prenyl transferases are of crucial importance in cell adaptation to a number of pathophysiological situations.

In the regenerating liver the regulation of the prenyltransferase enzymes appears to be exerted only on their activity, at least in the case of FTase and GGTase I. The results show that the activities of FTase and GGTase I progressively increase during the first cell cycle of liver regeneration. This increase in enzymatic activity is particularly intriguing considering the general increase of tissue protein content occurring during hepatic regeneration (23), while masses of FTase, GGTase I and GGTase II decreased in the first cell cycle.

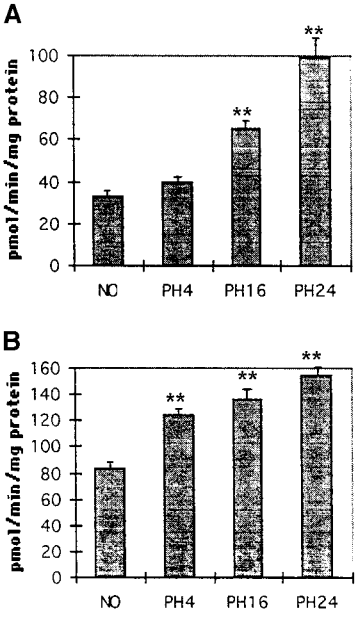

FIG. 2. FTase (A) and GGTase I (B) activity in liver cytosol of not operated (NO) or partially hepatectomized animals from 4 (PH4), 16 (PH16), and 24 (PH24) h. Results are reported as pmol ^{3}H-FPP or ^{3}H-GGPP bound/min/mg cytosolic protein (mean + S.D., $n = 3$ exp.). $^{**}P < 0.001$.

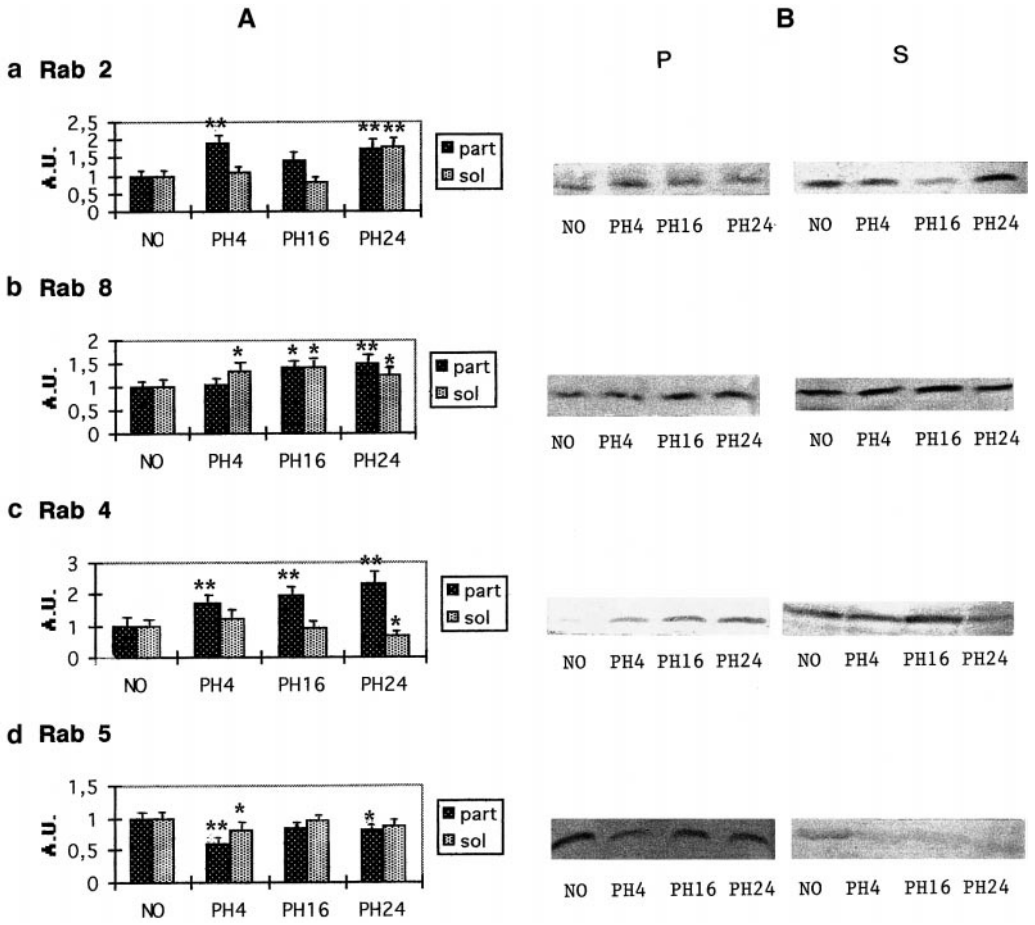

FIG. 3. Levels and subcellular distribution of Rab proteins. Rab 2, Rab 8, Rab 4, and Rab 5 were immunologically estimated in the particulate (P) and soluble (S) fractions of not operated (NO) or partially hepatectomized animals from 4 (PH4), 16 (PH16), and 24 (PH24) h. (A) Densitometric scanning of particulate (closed bars) and soluble (open bars) fractions: absorbance is expressed in arbitrary units (A.U.), with respect to NO set to 1 (mean + S.D., $n = 3$ exp.). *$P < 0.01$; **$P < 0.001$. (B) Representative immunoblot. Each lane contained 300 μg proteins.

It is likely that during hepatic regeneration, hepatocytes, which are heavily involved in many biosynthetic activities in preparation for mitosis, limit the biosynthesis of prenyl transferases, while increasing the activity of preexisting molecules. The higher activity of these enzymes could be necessary for the synthesis of prenylated proteins essential for cell division, as demonstrated in cultured cells (24). The increased amount of Rab proteins that was observed, would suggest enhanced GGTase II activity.

The variations in the levels of the Rabs are particularly interesting when considered in view of the relationships between vesicle intracellular traffic and the proliferative process. The hepatocytes, due to their position between plasma and bile, display very active vesicular trafficking of macromolecules (1). While much information is available about the uptake and secretion of these molecules, the machinery and regulation of intracellular trafficking in the liver have been studied less than in other tissues. This is partly because of the experimental difficulty in obtaining a polarized liver cell system "*in vitro*" for functional studies at the cellular level.

The regenerating liver exhibits metabolic variations related to the different phases of the cell cycle. This makes it a particularly good model to study the "*in vivo*" regulation of trafficking components. The processes of secretion of newly synthesized proteins are modified during regeneration. Hepatocytes in regenerating tissue exhibit decreased secretion of the blood proteins, such as albumin and apo B (25), as well as profound changes in bile acid secretion (26).

The finding that the levels of Rabs (2, 8 and 4) of the exocytic pathway increased during regeneration, during a period when protein secretion decreases, is suggestive of an increased need of exocytic insertion of resident molecules, such as carrier proteins, in the canalicular or sinusoidal membrane. The progressive increase of Rabs during the first cell cycle is probably essential in preparation to mitosis, to fulfill demands

related to the duplication of organelles. Rab levels also increased in parallel with proliferative activity in the developing chick embryo liver, another "*in vivo*" proliferating system (Bruscalupi *et al.*, unpublished results). Other data indicate overexpression of some Rabs in hematological tumors (27).

The decrease of Rab 5, that is thought to regulate the first stages of endocytosis, was unexpected. Earlier reports on hepatic regeneration indicated an increase in membrane ruffling, which was interpreted as an activation of endocytic processes (28). Actually, membrane ruffling has now been demonstrated to be independent on Rab 5 and endocytosis (29). The down-regulation of Rab 5 suggests that the regenerating liver limits the receptor-mediated uptake of extracellular molecules, and at the same time maintains the exposition of receptors for growth factors. Since the internalization process functions to down regulate signaling from growth factor receptors by removing receptors from plasma membranes (30), it is possible that the observed decrease of Rab 5 assures that transduction by growth factors is not impaired.

The distribution of the Rabs between membranes and cytosol is not significantly changed in the regenerating liver, with the exception of Rab 4, which shows a trend to increase in the particulate fraction and decrease in the soluble fraction. This suggests a progressive activation of Rab 4.

The independent pattern of each Rab protein indicates that each step of the vesicular traffic is independently regulated.

These results represent the first indication with "*in vivo*" liver of the regulation of Rab proteins dependent on cell proliferation

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