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Estrogen Stimulates Intracellular Traffic in the Liver of *Rana esculenta complex* by Modifying Rab Protein Content

G. Bruscalupi,*,[1] S. Cicuzza,* C. M. Allen,‡ L. Di Croce,*,[2] and A. Trentalance†

**Dipartimento di Biologia Cellulare e dello Sviluppo, Università "La Sapienza", Roma; †Dipartimento di Biologia, Università di Roma 3, Roma, Italy; and ‡Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida*

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During vitellogenesis in oviparous animals, estrogens induce the synthesis of the yolk precursor vitellogenin, a lipophosphoprotein rich in cholesterol. Estrogens also induce the activity of 3-hydroxy-3-methylglutaryl CoA reductase, that is necessary for the lipidation of vitellogenin. This increased enzyme activity could also be important for the production of isoprenoid groups that post-translationally modify proteins such as the Rab proteins, which are small G proteins involved in intracellular traffic.

The effect of estrogens on the production of prenylated proteins and on the levels of Rab proteins in the liver of *Rana esculenta complex* has been studied. An increase of the Rabs specifically involved in the exocytic pathway was observed and is probably related to the need for export of massive amounts of newly synthesized vitellogenin.

Key Words: **estrogen; amphibians; liver; vitellogenin; rab proteins; intracellular traffic.**

Amphibian liver is committed to the abundant synthesis and exocytosis of the yolk precursor vitellogenin (VTG). It is synthesized in the liver under the influence of estrogens and exported into the blood for delivery to the egg (1). Lipidation, phosphorylation, and glycosylation of VTG precede its secretion from the parenchimal liver cells. The massive amounts of VTG secreted imply a unique exocytic mechanism in the liver cell.

The proteins to be secreted move between different compartments of the secretory pathway. At each step, cargo is collected from one compartment into transport vesicles and delivered to the next compartment by fusion of the vesicles with the target compartment. This process is promoted and regulated by the Rab proteins, an evolutionary conserved family of low molecular weight GTP-binding proteins (2). They are post-translationally modified with prenyl groups that increase their hydrophobicity and favour their association with the membranes that is essential for their function. The prenylation is operated by a specific cytosolic enzyme, geranylgeranyltransferase II (GGTase II) or Rab prenyltransferase, that transfers one or two geranylgeranyl moieties to proteins (3). Rab proteins are suggested to serve as molecular switches for catalyzing the assembly of protein complexes involved in regulated assembly of the fusion machinery. They are thought to fulfill this function as they undergo a cycle of cytosol and membrane localization.

Each member of the Rab family is associated with a particular organelle. This gives support to the idea that Rabs play a role in the specificity of docking and fusion, although they are likely to exert their control via interaction with other proteins (4). Actually, each Rab seems to control a specific step in trafficking. Rab 5 is thought to regulate fusion of early endosomes (5); Rab 4 plays a role in the recycling pathway (6); Rab 1 and Rab 2 are required for ER to Golgi and intraGolgi transport (7, 8) and Rab 8 is thought to be involved in the exocytic pathway from trans-Golgi network (TGN) to plasma membrane (9).

Little is known about the possibility of regulating the trafficking machinery by hormones or other modulators. Well known is the insulin-mediated stimulation of glucose uptake by muscle cells and adipocytes. This occurs by translocation of vesicles containing the glucose transporter to the plasma membrane, a process dependent on Rab 4 (10). With regard to quantitative changes of the components of the trafficking machinery, there is an indication that the different endocytic capacity of hepatocytes and endothelial cells is related to a different content of the Rab proteins involved in endocytosis (11). Moreover in adipose cells, Rab 3D and

[1] To whom correspondence should be sent. Fax: 39-6-49912351. E-mail: bruscalupi@axcasp.caspur.it.
[2] Present address: IMT, University of Marburg, Germany.

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Rab 4 are up-regulated in genetic obesity, perhaps explaining the altered secretory properties observed in this state (12). In PC12 cells, the expression of Rab 3C is increased when cells are induced to differentiate in response to nerve growth factor or dibutyryl-cAMP(13). In spite of these few indications, it is not yet clear if trafficking apparatus is subject to regulation.

The estrogen-mediated secretion of vitellogenin in amphibian liver is a process that demands a massive need for trafficking machinery. Several GTP binding proteins in a molecular weight range similar to that of the Rabs have been identified in *Rana esculenta* liver (G.B., unpublished results), but the possibility that their synthesis or post-translational modification are regulated by estrogens is still unknown. On the other hand, several genes, among which amphibian VTG, are regulated by estrogens via an estrogen receptor (ER)-mediated pathway (14).

Therefore we have investigated the influence of estrogens on the levels of the Rab proteins involved in secretory and endocytic pathway in the liver of *Rana esculenta complex*. Our results suggest a correlation between estrogen administration and a modification of the levels and distribution of Rab proteins.

MATERIALS AND METHODS

Animals. Female frogs, *Rana esculenta complex* (25±5 g av.b.w.), were kept in an outdoor terrarium. Females were preferred to males because of their better responsiveness to hormone treatment (15). Estrogen-treated frogs were injected in the dorsal sac with 17α-ethynyl-estradiol (0,6 mg/ 25 g b.w.) in propylene glycol. The animals were sacrificed after anaesthesia with 0.2% MS222 (tricaine methane sulphonate) 4 hours, 1 day or 5 days after treatment. When tamoxifen was used, it was dissolved in propylene glycol and injected (3 mg/25g b.w.) in the dorsal sac 1 h before estrogens.When studying the influence of estrogens on protein prenylation, 2-14C-mevalonic acid (MVA) (5 μCi/25 g b.w.) was injected in the same way 15 hours before sacrifice at the end of a 3-day estrogen treatment. In each case livers were frozen in liquid nitrogen and stored at −80°C until use. Control animals received the same amount of carrier and were sacrificed at the same times. No effect of carrier alone was detectable. To avoid seasonal fluctuations, frozen livers collected at different year times from control or treated animals were pooled, and analyses were performed on multiple samples.

Mevalonate incorporation into prenylated proteins. Livers taken from animals injected with labelled MVA were homogenized in 10 volumes of PBS in the presence of 1 mM phenylmethylsulphonylfluoride (PMSF). Proteins were precipitated with 10 volumes of cold acetone and extensively delipidated according to Schmidt et al (16) in order to remove non-covalently bound lipids. Delipidated residues were solubilized overnight in 2% SDS, 10 mM Tris-HCl buffer, pH 7.4, at 37 °C. Samples (100-200 μg protein) were subjected to 10% SDS-PAGE according to Laemmli (17). For the estimation of protein-associated radioactivity, gels were sliced in 5 mm segments; each slice was solubilized with 1 ml Protosol (Packard):H_2O (9:1, v/v) at 60°C for 1 h and radioactivity determined in a Packard Tri-Carb scintillation counter.

Immunoblotting. The presence of Rab proteins and their membrane association was evaluated in particulate and soluble fractions from frog liver. Livers were homogenized in 25mM Tris HCl buffer pH 8.0 containing 1mM EDTA, 1 mM PMSF and 0.1 μM leupeptin. The 1000g post-nuclear supernatant was centrifuged at 140,000g for 90 min in order to obtain a membrane pellet (particulate fraction) and a cytosol supernatant (soluble fraction). The proteins in each fraction were resolved by 12.5% SDS-PAGE and transferred onto nitrocellulose paper. Rabs were revealed by immunoblotting with specific rabbit polyclonal antibodies (Santa Cruz Biotechnology)(1: 100); bound antibodies were visualized with alkaline phosphatase reaction, using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (Sigma) as substrate. Protein was quantitated by scanning densitometry of the membranes.

Immunoblotting of GGTase II β-subunit was performed on cytosol proteins separated by 12.5% SDS-PAGE. The primary antibody was raised (Cocalico Biologicals) in rabbit against a synthetic peptide Cys-Asp-Val-Thr-Ile-Lys-Ser-Asp-Ala-Pro-Asp (the N-terminal sequence of rat GGTase II β subunit) linked through the Cys to KLH. The positive antibody reaction was visualized as above.

Other methods. Proteins were measured by the method of Lowry (18) using bovine serum albumin as a standard. All the images were quantitated by scanning densitometry using a NIHImage 1.51 program with a Macintosh computer.

RESULTS

The incorporation of labeled mevalonate into prenyl groups covalently bound to proteins was evaluated in the liver of frogs treated with a single dose of estrogens for 3 days. Estrogen treated frogs showed a modest increase (30%) in the incorporation of mevalonate into the liver tissue and delipidated liver proteins as compared to control (Table I). However, examination of the M.W. distribution of the prenylated proteins in the estrogenized frogs shows a dramatic increase (a 7-fold increase over the control) in labeling of proteins in the 20-30 kD range (Fig.1, Table I). This is the M.W. range represented by the Rab proteins. These results suggest that estrogens have a marked effect on the biosynthesis of MVA-derived proteins.

Next, the amount of several Rab proteins in the liver of *Rana esculenta* were estimated under basal conditions and after estrogen stimulation. We choose Rab 1, 2 and 8 as markers of the secretory pathway and Rab 4 and 5 as markers of the endocytic-recycling pathway. Proteins were isolated 4 hours, one day and five days after estrogen administration. The short time was chosen because estrogens are known to induce transcriptional effects on some genes, such as that of VTG, as early as two hours after hormone administration (19). The later time was chosen to correspond to the maximal induction of HMGCoA reductase activity, the key enzyme in the synthesis of prenyl groups, after estrogen treatment (20). Rab proteins were estimated in both particulate and soluble liver fractions in order to obtain information about the proportion of Rab proteins in the active state (i.e. membrane associated).

The levels of Rab 1, which is involved in the transport between ER and cis-Golgi, are already significantly and precociously increased in estrogenized frogs at 4 hours after treatment (Figure 2a). The increased protein is more highly represented in particulate fraction but the increase is also appreciable on the soluble

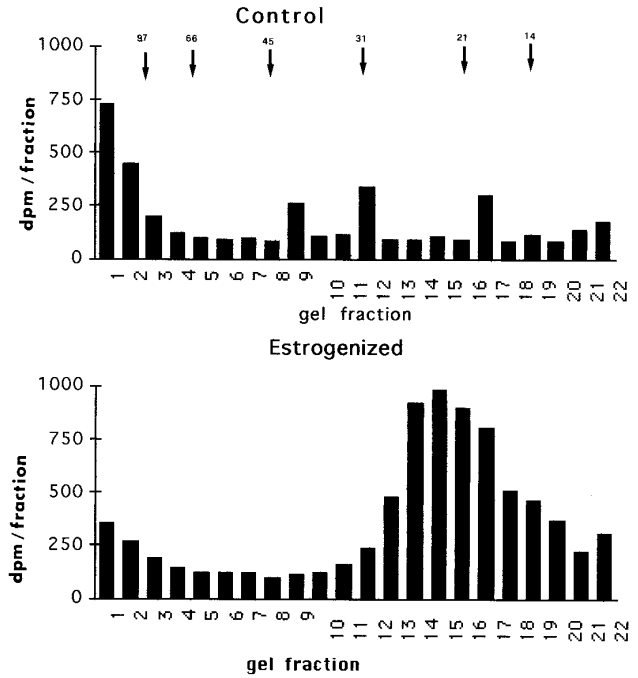

FIG. 1. Distribution of mevalonic acid labeled liver proteins from control and estrogenized frogs. 2-14C-MVA-labeled proteins were isolated from the livers of untreated (panel A) or estrogenized (panel B) frogs treated for 3-days as described in Table I. Proteins were separated by 10% SDS-PAGE and analyzed for radioactivity. Arrows indicate the position of M.W. standards.

fraction. Therefore the mass of Rab 1 increases following estrogen stimulation, whereas the activation state is not changed.

The levels of Rab 2, which is also involved in ER-Golgi transport, are increased more gradually in the particulate fraction after estrogen treatment, with a peak at one day which is double than the control. On the other hand, the protein levels in the soluble fraction decrease progressively and become one-half the basal level at 5 days (Fig. 2b). This suggests a redistribution of Rab 2 to the membrane.

The third protein of the secretory pathway considered is Rab 8, which is involved in protein transport from trans-Golgi network to the basolateral membrane in epithelial cells. This protein is increased particularly strongly by estrogen treatment, especially in the particulate fraction; therefore both a mass increase and a redistribution is likely to occur in this case (Fig. 2c).

In order to evaluate the recycling pathway, Rab 4 has been examined (Fig. 2d). This protein displays a peculiar behaviour, since it is almost exclusively present in the soluble fraction both in the unstimulated and in the estrogenized animals, and exhibits a difference from the distribution in the rat, where the bulk of Rab 4 is in the membrane fraction (G.B., unpublished results). Moreover, the portion of protein residing in the soluble fraction is further decreased by estrogens, and the low level of the particulate fraction is not significantly modified by hormones.

The marker of the endocytic pathway, Rab 5, is less represented or more difficult to detect than other members of the Rab family; however, it is present in both the subcellular fractions. After estrogen treatment, the amount of Rab 5 in the particulate fraction undergoes a three-fold increase that persist for while, then decreases by 5 days. Also the soluble Rab 5 is increased, with a peak at 1 day (Fig. 2e).

To determine if estrogens exert a direct influence on Rab protein expression via a receptor-mediated process, frogs were treated *in vivo* with tamoxifen along with estrogens for three days and Rab 1 expression was measured. Tamoxifen inhibits the nuclear actions of the estrogens by binding to the cytosolic receptor. Rab 1 was tested because its expression is strongly sensitive to estrogens. Fig. 3 shows that Tamoxifen strongly reduces the induction of Rab 1 by estrogen, suggesting that at least part of the induction is mediated by receptor.

In order to evaluate the possibility that estrogens might also increase the prenylation activity of the frog liver, we have tested the presence of GGTase II in the cytosol. An antibody against the rat β-subunit recognizes a protein band in frog liver which has a M.W. slightly higher than the band found in the rat liver (39.5 kD against 38 kD). No significant difference in the level of this prenyltransferase subunit was observable after estrogen treatment (intermediate times not shown) (Fig. 4).

DISCUSSION

Rab proteins are key regulators of vesicular traffic (21). The current studies were performed to establish if the amount of these proteins is modified during massive trafficking. We compared the levels of Rab proteins in the liver of normal and estrogen-treated frogs, where the secretion of vitellogenin is very high. Until now, observations regarding hormonal dependence of vesicular traffic were related to the hormone-triggered exposition of vesicles containing rapid-deliverable pre-

TABLE I

Mevalonic Acid Labeling of Frog Liver Proteins

Animals	dpm/gr tissue (1)	dpm/gr delipidated tissue (2)	dpm/20-30 KDa gel fractions (3)
C	8,437,500	830,272	4,830 (0.06% of 1)
E	11,467,500	1,025,000	28,637 (0.28% of 1)

Note. 2-^{14}C-MVA (1.5×10^7 dpm, 50 mCi/mmol) was injected into control (C) and 3 days-estrogenized (E) frogs 15 h before sacrifice. Total liver (1), extensively delipidated proteins (2) and proteins in the 20-28 kDa range (3) were analyzed for radioactivity. Data are representative of two independent experiments.

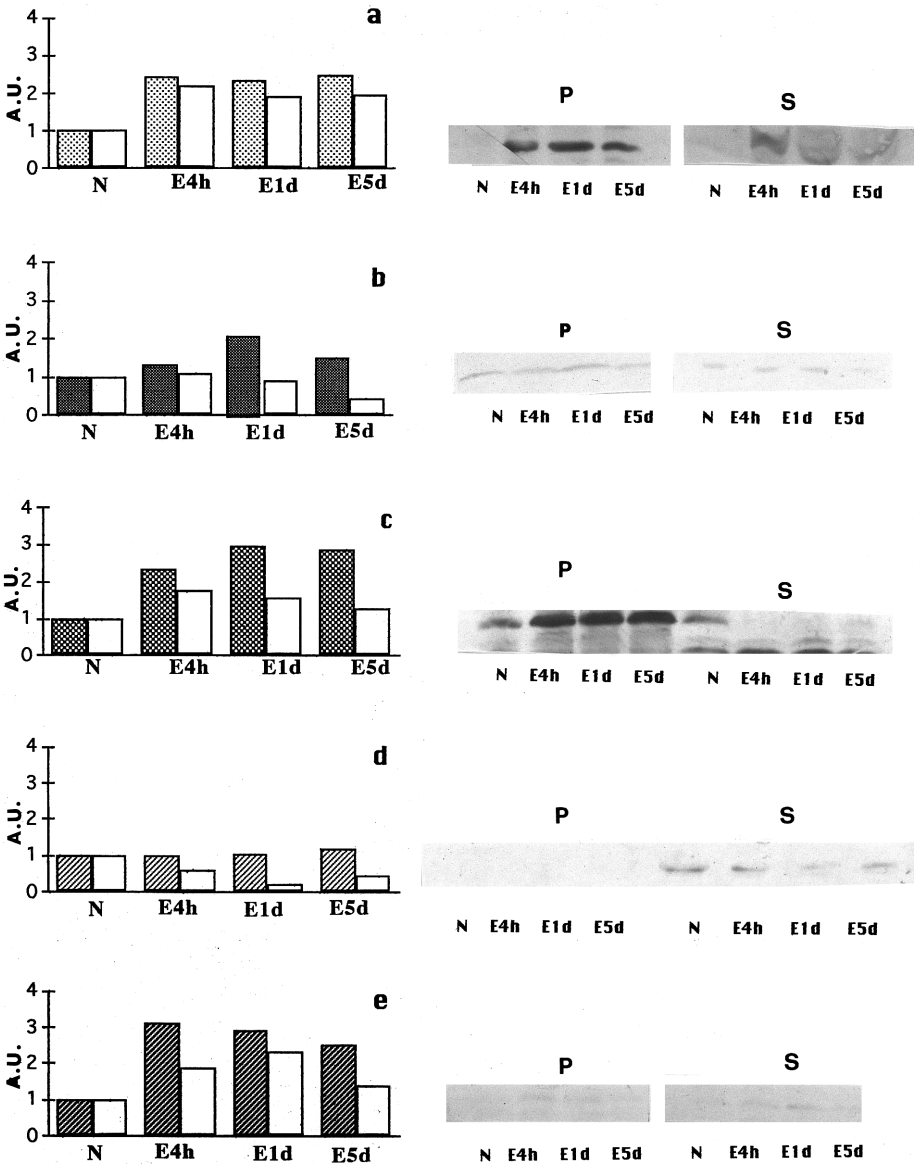

FIG. 2. Levels and subcellular distribution of Rab proeteins. Rab 1(a), 2 (b), 8 (c), 4 (d) and 5 (e) were estimated immunologically (see Materials and Methods) in the particulate (P) and the soluble (S) fractions of the liver of control (N) and estrogenized (E) frogs. **A.** Representative immunoblot. Each lane contains 300 ug proteins. **B.** Densitometric scanning of particulate (closed bars) and soluble (open bars) fractions: absorbance is expressed in arbitrary units (A.U.), with respect to non estrogenized frog set to 1 (mean + S.D., n=3 experiments).

formed cargo to the cell surface (10, 22). Steady state levels of Rab proteins are generally believed to be quantitatively sufficient for cellular requirements.

In the case we examined, where estrogens dramatically increase the amount of cargo to be secreted, we have observed that all the Rabs involved in the exocytic-secretory pathway are increased or at least activated. This is especially true for Rab 8, a controlling protein in the last step of exocytosis.

The first suggestion that estrogens affect Rabs was the observation that these hormones dramatically increased the incorporation of MVA-derived radioactivity in low M.W. proteins. The increase in prenylation could be due to an increased prenylation of preexisting Rabs, or to increased mass of Rabs. The first hypothesis is apparently ruled out, since the prenyltransferase mass does not change (Fig. 4), and it is generally accepted that all mature Rabs are irreversibly prenylated (23).

Changes in mass were investigated for each individual Rab and increases were ascribable to only some of the proteins tested, in particular all those involved in exocytosis. On the contrary levels of Rab 4, involved in recycling, were not modified. Therefore the results show that estrogen treatment has the general effect of

changing the levels of the Rab proteins but with different timing and different directions.

However, it is likely that coordination exists between all the steps of vitellogenin secretion. That is: **i)** VTG gene transcription is induced by estrogens in Xenopus (14) and avian (24) liver; **ii)** estrogens increase the mass and activity of the key enzyme of cholesterol biosynthesis, HMGCoA reductase, in Xenopus (25) and Rana esculenta (20) liver. This enzyme is responsible for the lipidation of newly synthesized vitellogenin (26); **iii)** estrogens induce the levels of the Rab involved in the secretory pathway; **iiii)** the uptake of plasma VTG into the oocytes is also facilitated by estrogens (27).

It remains to be clarifyed if the Rab levels are increased directly by estrogens, or if the modification is secondary to the increased cargo. The partial inhibition of estrogen-induced increase of Rab 1 in tamoxifen-treated frogs suggests that the hormonal effect is at least partially receptor-mediated. It has been known for a long time that the effect of estrogens on VTG gene trancription occurs at an ERE (14), whereas the effect on HMGCoA reductase is likely a consequence of post-transcriptional regulation (20). No information is yet available about the presence of an ERE in amphibian Rab genes.

Estrogens also markedly enhance the overall protein synthetic capacity of the amphibian liver (28), but the increase of only some members of the Rab family suggests a more specific effect.

The increased levels of Rab proteins induced by estrogens are not paralleled by an increase of GGTase II, the enzyme involved in their post-translational modification. Since the increased need of prenyl groups is met by the enhanced activity of HMGCoA reductase (also induced by estrogen treatment)(25), the data suggest that the preexisting levels of GGTase II are sufficient to manage the process. However, an increase of the activity cannot be ruled out.

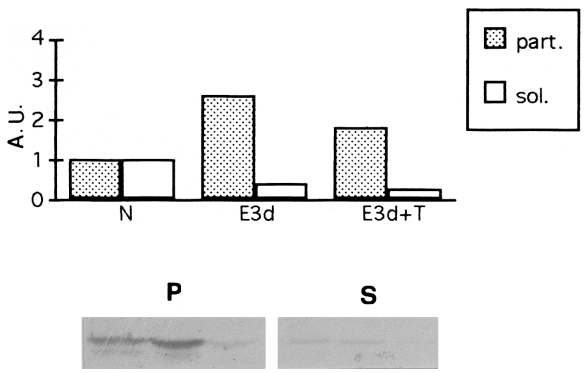

FIG. 3. Immunoblot of Rab 1 in particulate (P) and soluble (S) fraction from livers of not-estrogenized (N), estrogenized (E) and tamoxifen+estrogen-treated frogs (T). Each lane contained 300 ug proteins. (n=2 experiments).

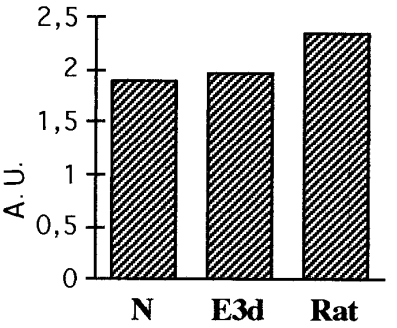

FIG. 4. Rab GGTase determination in liver cytosol from control (N) or 5 days-estrogenized frogs (E5). Rat liver cytosol was used as a positive control. **A.** Representative immunoblot. Each lane contains 200 ug proteins. **B.** Densitometric scanning: absorbance is expressed in arbitrary units (A.U.), with respect to non estrogenized frog set to 1 (mean + S.D., n=3 experiments).

In conclusion, for the first time we have shown that estrogens have an effect on Rab protein levels. This effect, specifically targeted to Rab 1, 2, and 8, leads to an increased activity of the exocytosis pathway. The data suggest that estrogens regulate gene transcription of Rab proteins via the estrogen receptor.

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