Phosphatidylinositol 3-Kinase Translocates onto Liver Endoplasmic Reticulum and May Account for the Inhibition of Glucose-6-phosphatase during Refeeding*

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By using a rapid procedure of isolation of microsomes, we have shown that the liver glucose-6-phosphatase activity was lowered by about 30% (p < 0.001) after refeeding for 360 min rats previously unfed for 48 h, whereas the amount of glucose-6-phosphatase protein was not lowered during the same time. The amount of the regulatory subunit (p85) and the catalytic activity of phosphatidylinositol 3-kinase (PI3K) were higher by a factor of 2.6 and 2.4, respectively (p < 0.01), in microsomes from refed as compared with fasted rats. This resulted from a translocation process because the total amount of p85 was the same in the whole liver homogenates from fasted and refed rats. The amount of insulin receptor substrate 1 (IRS1) was also higher by a factor of 2.6 in microsomes from refed rats (p < 0.01). Microsome-bound IRS1 was only detected in p85 immunoprecipitates. These results strongly suggest that an insulin-triggered mechanism of translocation of PI3K onto microsomes occurs in the liver of rats during refeeding. This process, via the lipid products of PI3K, which are potent inhibitors of glucose-6-phosphatase (Mithieux, G., Danie`le, N., Payrastre, B., and Zitoun, C. (1998) J. Biol. Chem. 273, 17-19), may account for the inhibition of the enzyme and participate to the inhibition of hepatic glucose production occurring in this situation.

Glucose-6-phosphatase (Glc6Pase) is a crucial enzyme in systemic glucose homeostasis. By catalyzing the dephosphorylation of glucose 6-phosphate (Glc6P), e.g. the last biochemical reaction of glycogenolysis and gluconeogenesis, it allows the gluconeogenic tissues (liver and kidney) to release glucose in the blood (1). Numerous data have been recently provided suggesting that Glc6Pase is an important regulatory factor of glucose production by the liver and the kidney in various physiological situations through mechanisms involving either gene expression (2–5) or inhibitions of its enzymatic activity (Refs. 6–9, recently reviewed in Ref. 10). The inhibition of the Glc6Pase activity during the postprandial period was suggested more than 10 years ago (11). This inhibition has remained uncertain for a long time because the biochemical evidence for the inhibition of the enzyme activity in the test tube was lacking. A significant breakthrough has been recently achieved, as we have shown that the Glc6Pase activity, assayed in the homogenates from rat livers freeze-clamped in situ, is inhibited for a few h after refeeding (9). Other indirect data have strongly suggested that hyperinsulinemia, associated or not to hyperglycemia, should be the key signaling factor in the inhibition mechanism (10, 12, 13). However, the inhibition was not observed in the liver homogenates from rats perfused with insulin, whereas euglycemia was maintained by glucose perfusion (9). This might be explained because the latter conditions dramatically differ from the physiological ones. We have therefore chosen to study the mechanism of inhibition of Glc6Pase under physiological conditions of hyperinsulinemia, e.g. during the refeeding of rats previously unfed for 48 h (9). A key feature of this mechanism is its lability because the inhibition was not retained in isolated microsomes in any study (6, 9, 10, 13). This was a serious hindrance to the elucidation of the mechanism at the molecular level. Our first aim has, thus, been to design a purification procedure of microsomes retaining the inhibited state of the enzyme.

In parallel, we have wished to assess the hypothesis that the inhibition of Glc6Pase could be dependent on the activity of phosphatidylinositol 3-kinase (PI3K). There is indeed growing evidence suggesting that PI3K is a crucial enzyme in the signal transduction of insulin to glucose metabolism in the liver and in peripheral tissues (see Refs. 14 and 15 as reviews). Suggesting a role for PI3K in the control of Glc6Pase activity, we have recently shown that the enzyme is specifically inhibited in vitro in the presence of μM amounts of the lipid products of PI3K (16). In adipocytes, it has been strongly suggested that the specificity of insulin to stimulate glucose transport is dependent on the intracellular targeting of PI3K to low density microsomes (17–19). Given the intracellular location of Glc6Pase in the liver endoplasmic reticulum (which is not explained at the present time), the hypothesis that a similar PI3K translocation process could be involved in the Glc6Pase inhibition seems therefore especially attractive.

MATERIALS AND METHODS

Refeeding Experiments—Male Sprague-Dawley rats (IFAA CREDO, L'Arsbrele, France) weighing 230–240 g were housed for 3 days with free access to water and rat chow (50% starch, 23.5% proteins, 5% lipids, 4% cellulose, 5.5% mineral salts, 12% water (weight basis), Unité d'Alimentation Rationnelle, Epinay/Orges, France). Fasted rats were then deprived of food for 48 h with free access to water. After the same fasting time, refed rats were given free access to rat chow (see above) for 360 min (9). The results were obtained from several sets of experiments. Each set involved one control group of three fasted rats and one group of three refed rats processed the same day.

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**Rapid Liver Subcellular Fractionation**—Unfed and refed rats were anesthetized by a single injection of pentobarbital (7 mg/100 g of body weight). Once they were asleep, the abdomen was incised to expose the liver. A small liver lobe (about 2 g) was cut and immediately homogenized at 4 °C in 18 ml of 10 mM Hepes, 0.25% sucrose, pH 7.3, using a Teflon glass homogenizer. The homogenate was rapidly centrifuged for 10 min at 4 °C at 30,000 × g to pellet all dense intracellular vesicles (20). Microsomes were further extracted from the supernatant by one centrifugation step for 30 min at 100,000 × g at 4 °C. The microsomal pellet was resuspended in 1.5 ml of the Hepes/sucrose buffer and was immediately used for Glc6Pase and PI3K assays. Using this procedure, less than 1% of the protein was lost between the removal of the liver lobe and the enzyme assays. In some experiments, a further fractionation of microsomal membranes in sucrose gradient was performed according to the method described by Aronson and Touster (21). The rest of the microsomes were frozen and kept at −80 °C for subsequent determination of protein and Western blot analyses. In some experiments, microsomes were also isolated according to the classical procedure described in our previous papers (4, 7).

**Enzyme Assays**—Glc6Pase was assayed after rapid fractionation by complex formation of P, produced from Glc6P. Brieﬂy, the microsome suspension was diluted 50 times in 10 mM Hepes, 0.25% sucrose, pH 7.3, and 100 μl (about 30 μg) of protein were added to a 500-μl (total volume) incubation medium composed of 20 mM Tris-HCl, 1 or 20 mM dithiothreitol, 100 mM NaCl, 100 μM vanadate, 50 μM ATP (20 μCi [γ-32P]ATP), 15 μg of phosphatidylinositol-4,5-bisphosphate (Sigma) and 30 μg of phosphatidylylycerol (Sigma), pH 7.3. After a 15-min incubation at 37 °C under agitation, the reaction was stopped by the addition of 2 ml of acetic acid/trichloroacetic acid (2%/10%, mass/vol), and P was determined as described (5, 7, 9). PI3K was assayed essentially as described previously (22), with slight modiﬁcations. Brieﬂy, 5 μl of the suspension of microsomes (about 75 μg of proteins) were diluted in a 70-μl incubation medium (total volume) composed of 50 mM Tris-HCl, 5 mM MgCl2, 0.5 mM EDTA, 1.5 mM dithiothreitol, 100 mM NaCl, 100 μM vanadate, 50 μM ATP (20 μCi [γ-32P]ATP), 15 μg of phosphatidylinositol-4,5-bisphosphate (Sigma) and 30 μg of phosphatidylylycerol (Sigma), pH 7.3. After a 15-min incubation at 37 °C under agitation, the reaction was stopped by the addition of 400 μl of chloroform/methanol (1:1, vol:vol). Phospholipids were extracted and deacylated, and [32P]glycerophosphoinositol-3,4,5-trisphosphate was quantified by HPLC technique as described previously (23, 24). 5′-Nucleotidase was determined according to a previously published procedure (21).

**Western Blot Analyses**—A rabbit polyclonal antiserum against rat Glc6Pase was obtained by injection of a synthetic peptide matching the 14 amino acids of the C terminal end of Glc6Pase sequence (CLARLL-QTHHKSL) coupled to keyhole limpet hemocyanin. A rabbit polyclonal antiserum against the regulatory subunit (p85) of rat PI3K and a rabbit immunopurified IgG fraction raised against rat insulin receptor substrate 1 (IRS1) were obtained from Euromedex (Souffleweyer-Sheim, France). Microsomal proteins were subjected to electrophoresis on 9% polyacrylamide gels in the presence of SDS and transferred to Immobilon-P membranes (Millipore S.A., St. Quentin sur Yvelines, France). After blocking, the membranes were incubated with either anti-Glc6Pase antiserum (1:500 dilution), anti-PI3K antiserum (1:1000), or anti-IRS1 IgG (1:1000) followed by incubation with a pure goat-IgG fraction directed against rabbit IgG and linked to peroxidase (1:10000) (Sigma). The detection was performed using a speciﬁc chemiluminescence system (Serva S.A., Ste-Foy-les-Lyon, France) with ECL-hyperﬁlms from Amersham Pharmacia Biotech.

**Other Methods**—Protein was assayed according to the procedure of Lowry with bovine serum albumin as a standard in all experiments (25), with the exception of membrane subfractions from sucrose gradients, which were assayed according to the Bradford procedure with the same standard (26). Statistical analyses were performed using the Student’s t test for unpaired data (27).

**RESULTS AND DISCUSSION**

The challenging problem in studying the molecular mechanism of Glc6Pase inhibition after refeeding was to achieve the isolation of microsomes with Glc6Pase retaining its inhibited state. In a first approach, we tried to homogenize the liver and to purify the microsomes in the presence of insulin mimetics such as orthovanadate and its powerful derived compounds, i.e. aqueous huperoxovonadate and huperoxonvandium picolinate. Unfortunately, this approach could not be used because, after homogenization of the liver in the presence of either of the vanadate compounds, Glc6Pase activity was revealed to be unstable and to decrease progressively with time, even if the inhibitors were rapidly removed upon washing microsomes (not shown). Therefore, we tried to obviate the problem of the lability of Glc6Pase inhibition by using a rapid procedure of extraction of microsomes from the liver homogenates (see “Materials and Methods”). Using this approach, we demonstrated that microsomal Glc6Pase activity was inhibited after refeeding, when it was assayed at low concentration (1 μM) of substrate (65 ± 2 versus 96.5 ± 3 nmol/min/mg of protein (33% inhibition)) and at high concentration (20 μM) of substrate (225 ± 6 versus 305 ± 9 nmol/min/mg of protein (26% inhibition) (see Fig. 1)). This was in good agreement with our previous results involving Glc6Pase assays in the homogenates from livers freeze-clamped in situ in anesthetized refed rats (9). The inhibition was because of an inhibition of the enzyme activity and not to a decrease in the amount of protein because there were no differences regarding to the amounts of immunoreactive 36-kDa protein (Glc6Pase), irrespective of the isolation procedure of microsomes or of the nutritional status of the animals (upper panel of Fig. 1). This conﬁrmed our previous data reporting that there was no detectable decrease in Glc6Pase activity after refeeding when the enzyme was assayed in microsomes isolated using the classical procedure (9).

On the basis of this result, we were able to test the hypothesis that a mechanism of translocation of PI3K could be involved in the inhibition mechanism of Glc6Pase. In agreement with such a hypothesis, both the amount of immunoreactive p85 and the catalytic activity of PI3K were enhanced by 2.6 and 2.4 times, respectively, in the microsomes isolated from refed rats as compared with fasted rats (Fig. 2 A and B). This increase was the consequence of a translocation process and not of a global increase in the amount of p85, because the amount of immunoreactive p85 in the homogenates from total livers was similar in refed rats and in fasted rats (Fig. 2 D).

From microsomal protein recovery determinations and compar-
isons of the amounts of immunoreactive p85 in microsomes and homogenates, we could estimate that p85 bound to microsomes represented about 10–15% of total liver p85 in fasted rats and about 25–30% in refed rats. In 3T3-L1 adipocyte cells, it has been strongly suggested that the specificity of insulin to stimulate glucose transport is dependent on its ability to target PI3K to low density microsomes, from which glucose transporters translocate (18). In contrast, platelet-derived growth factor, which does not stimulate glucose transport, mainly activates p85 along the gradient was comparable with that in refed rats (Fig. 3). This suggested that the inhibition from refed rats as compared with the corresponding fractions from refed rats (Fig. 2, A and C). Noteworthy, Glc6Pase activity was significantly lower in fractions 6 to 8 (endoplasmic reticulum) from refed rats as compared with the corresponding fractions from fasted rats (Fig. 3). This suggested that the inhibition was stable after microsomes had been rapidly extracted from the liver homogenates. In contrast, it was not lower in fractions 2 to 4 (plasma membranes) in which a substantial proportion of Glc6Pase activity might be accounted for by the presence of nonspecific phosphatase activities such as alkaline phosphatase.

It is now admitted that phosphatidylinositol-3,4-bisphosphate and, more likely, phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5P3) are the key lipid metabolites of the PI3K-signaling pathway, because they are almost absent from resting cells, whereas their intracellular concentration substantially increases upon stimulation (29). Previously, we performed reconstitution experiments of microsomes purified from
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FIG. 3. Subfractionation of liver microsomal membranes on sucrose gradients. Liver microsomes rapidly isolated from fasted (open symbols) and refed rats (closed symbols) were subfractionated in 11-ml (total volume) sucrose gradients. 5′-Nucleotidase activity (squares) was determined to identify the plasma membranes (top of the gradient), and Glc6Pase (circles) was determined to identify the endoplasmic reticulum (medium part of the gradient). The results are expressed as the mean ± S.E. (n = 3). *, significantly different from the corresponding fasted value. The upper panel shows the immunodetection of p85 in the fractions 2 to 10 (fractions 1 and 11 were not analyzed because of very low total protein content) from a subfractionation experiment of liver microsomes from refed rats (15 μg of protein analyzed in each lane). The experiment shown is representative of three yielding comparable results.

Fasted rats using our classical procedure in which Glc6Pase is fully active, with pure phosphatidylinositol-3,4-bisphosphate and PI3,4,5P₃. We have shown that Glc6Pase activity is inhibited in vitro in the presence of minute amounts of both D3-phosphoinositides (PI3,4,5P₃ being the most efficient inhibitor) and not in the presence of various other phospholipids (16). To document in vivo that the products of translocated PI3K are the inducing factor in the Glc6Pase inhibition, we quantified PI3,4,5P₃ in microsomes from fasted and refed rats by two approaches. The one involved the metabolic labeling of the ATP pool by 32P in rats before refeeding experiments and the analysis of [32P]glycerophosphoinositol-3,4,5-trisphosphate by HPLC (see “Materials and Methods”). The other involved the separation of phosphoinositides by thin layer chromatography and quantification of PI3,4,5P₃ by densitometry. Unfortunately, neither approach allowed us evidence of a higher total PI3,4,5P₃ content in microsomes from refed rats as compared with fasted rats (data not shown). Although this is not a positive result, we would like to point out that it does not exclude that PI3,4,5P₃ is the inhibitor of Glc6Pase in refed rats. Indeed 1) the PI3,4,5P₃ fraction bound to (and inhibiting) Glc6Pase should likely represent only a minor fraction from the total microsomal PI3,4,5P₃ content; 2) the bulk microsomal PI3,4,5P₃ content might have returned to its basal fasting level because of the action of counter-regulatory phosphoinositide phosphatases during the isolation procedure; 3) those PI3,4,5P₃ molecules inhibiting Glc6Pase could be protected from the action of phosphoinositide phosphatases because of tight binding to the enzyme. We have previously suggested that the binding of the lipidic part of the PI3,4,5P₃ molecule to some hydrophobic region of the enzyme is able to stabilize the enzyme-inhibitor interaction (16). One may thus hypothesize that this particular PI3,4,5P₃ pool bound to Glc6Pase does return to basal level with a lengthened time course as compared with the bulk microsomal PI3,4,5P₃ content. This might explain why the Glc6Pase inhibition did not decrease in parallel with the total inhibitor concentration as the isolation of microsomes was ongoing. Alternatively, the reversibility of the PI3,4,5P₃-mediated Glc6Pase inhibition might involve the action of a regulatory molecule that is not present in rapidly isolated microsomes. That the Glc6Pase inhibition seemed stable in the endoplasmic reticulum after fractionation in sucrose gradients (Fig. 3) is in agreement with the latter proposal.

To obtain another type of confirmation of the role of PI3K in the Glc6Pase inhibition in vivo, we carried out refeeding experiments with rats treated by intraperitoneal injections of wortmannin, a specific inhibitor of PI3K (14). A similar approach was successfully used to inhibit skeletal muscle P70 S6 kinase by rapamycin in mice (30). Four injections (one every 2 h from 1 h before refeeding) were given at doses of 100 μg/kg by intraperitoneal injection. This represented a total dose close to the maximally tolerated daily dose in mice (31). However, the liver microsomal PI3K activity was not substantially inhibited under these in vivo conditions (by about 30% only, data not shown), and Glc6Pase activity was inhibited in wortmannin-treated refed rats as in control saline-injected refed rats (61 ± 4 versus 63 ± 4 nmol/min/mg of protein at 1 min Glc6P and 237 ± 16 versus 228 ± 2 nmol/min/mg of protein at 20 min, respectively, compared with the results of Fig. 1). Another approach was successfully used with LY294002, another specific inhibitor of PI3K (4 injections as above at 25 mg/kg/injection), which was also unsuccessful in relieving Glc6Pase activity from inhibition (not shown). Unfortunately, we could not obtain this additional confirmation for a role of PI3K in the control of Glc6Pase activity. Again, we would like to emphasize that this result is not counter to the basic proposition that PI3K is responsible for Glc6Pase inhibition. It is likely that a 30% inhibition only of PI3K activity might not be sufficient to alter the inhibition of Glc6Pase significantly. In addition, it cannot be excluded that the PI3K isotype involved in the control of Glc6Pase is insensitive to the inhibitors used in this work. PI3K species insensitive to one or both of these inhibitors have indeed been described (32–34).

A remaining important question is to know why Glc6Pase inhibition could only be evidenced in the most physiological situation of hyperinsulinemia, e.g. rats refeed after a period of fasting and not in rats perfused with insulin (9), whereas compelling indirect evidence has been provided that insulin inhibits hepatic glucose production by acting on Glc6Pase (10, 12, 13). This could be explained by a differential stability of Glc6Pase inhibition, which could be accounted for by the presence of a co-factor during refeeding experiments and its absence during insulin perfusion experiments. This might explain why isolated hepatocytes have often revealed a weakly suitable model to study the short term metabolic effects of insulin in the liver. We have previously discussed the possibility that some liver metabolite(s) dependent on nutrient availability could be required together with insulin for a full inhibition of Glc6Pase to take place in vivo (9, 10). Noteworthy, recent results from us (35) and others (36) have emphasized the crucial role that hyperglycemia could play in the inhibition of the hepatic Glc6Pase flux in vivo. Efforts are ongoing to identify the putative factor(s) required for the inhibition mechanism of Glc6Pase under the action of insulin to be clearly demonstrated in a simplified experimental model. Such a demonstration in isolated hepatocytes, for example, would allow us to question the role of PI3K by means of PI3K inhibitors.

In conclusion, we report the definitive demonstration in isolated microsomes that a mechanism of inhibition of Glc6Pase activity takes place in the liver of rats during the postprandial period. In addition, our results strongly suggest that this inhibi-
bition mechanism could be dependent on a translocation process of PI3K onto the liver endoplasmic reticulum membranes, a likely consequence of the activation of the insulin-signaling pathway, and mediated by the main lipid product of PI3K, e.g. PI3,4,5P₃. Together with the recent reports that the targeting of PI3K to intracellular membranes could be a key mechanism in the activation of glucose transport by insulin in isolated adipocytes (15, 17–19) and in the inhibition of apolipoprotein B secretion by insulin in isolated hepatocytes (37), the data presented here further document in vivo that translocation processes of PI3K might constitute a general process in insulin signaling in both the liver and peripheral tissues. In the liver, they bring new insights into the mechanisms of control of hepatic glucose production at the level of Glc6Pase and the involvement of PI3K in these processes. They also provide a new rationale regarding the impairment of insulin in suppressing hepatic glucose production in insulin-resistant animals, because the IRS1-PI3K activation pathway is strongly altered in the liver of such animals (14, 38, 39).

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