Evidence for a Membrane Exchangeable Glucose Pool in the Functioning of Rat Liver Glucose-6-phosphatase*

(Received for publication, April 4, 1995, and in revised form, July 12, 1995)

Alfred Berteloot‡§, J. Jean-François St-Denis¶, and Gérald van de Werve††

From the ‡Laboratoire d’Endocrinologie Métabolique, Departments of Nutrition and Biochemistry, the ¶Groupe de Recherche en Transport Membranaire, and the ††Department of Physiology, Université de Montréal, Montréal, Québec H3C 3J7, Canada

We have investigated the kinetics of tracer uptake into rat liver microsomes in relation to [14C]glucose 6-phosphate (Glu-6-P) hydrolysis by glucose 6-phosphatase (Glu-6-Pase). 1) The steady-state levels of intravesicular tracer accumulated during the rapid (AMP1) and slow (AMP2) phases of uptake both demonstrate Michaelis-Menten kinetics relative to outside Glu-6-P concentrations with Km values similar to those observed for the initial burst (V1) and steady-state (Vss) rates of Glu-6-P hydrolysis. 2) The AMP1/AMP2 ratio is constant (mean value = 0.105 ± 0.018) over the whole range of outside Glu-6-P concentrations and is equal to the AMP1max/AMP2max ratio (0.109 ± 0.032). 3) Linear relationships are observed between the initial rates of glucose transport across the slow uptake phase (Vss) and [AMP1], and between [Vss] and [AMP2]. 4) The value of Vss exceeds by more than 10-fold that of V1 (Vssmax > V1max), and Vssmax is incompatible with those results and that AMP1 represents a membrane exchangeable glucose pool. 6) We propose a new version of the conformational model in which the catalytic site lies deep within a hydrophilic pocket of an intrinsic membrane protein and communicates with the extra- and intravesicular spaces through channels with different glucose permeabilities.

The localization of glucose 6-phosphatase (Glu-6-Pase) (EC 3.1.3.9) within the membrane of the endoplasmic reticulum (ER) has led to consider that glucose 6-phosphate (Glu-6-P) hydrolysis in vivo should be associated with transport functions allowing both substrate and products to cross the ER membrane. In agreement with that idea, Glu-6-Pase activity was found to be increased in disrupted rat liver microsomes as compared with intact vesicles, and the enzyme activity has therefore been described as latent (1, 2). The basis of Glu-6-Pase latency has led to much controversy in the literature, and two theories have emerged during the last 20 years. In the substrate transport model (1–7), it is postulated that the enzyme is part of a multicomponent system in which the catalytic site is oriented toward the lumen of the ER and associated with specific transport proteins for Glu-6-P (T1), phosphate (T2), and glucose (T3) (4). Accordingly, Glu-6-Pase latency has been interpreted as evidence for a rate-limiting step in Glu-6-P transport through T1. In contrast, the conformational model (8–10) proposes that Glu-6-Pase is a single entity sequestered within the ER membrane. The catalytic site is thus freely accessible from the cytoplasm, and Glu-6-Pase latency would result from changes in the membrane environment that modify the interactions of the enzyme with its substrates and products.

In agreement with the conformational model, our recent kinetic studies using a fast-sampling, rapid-filtration apparatus (FSRFA, U. S. patent 07/697,769) have brought evidence for a tight coupling between Glu-6-P transport and Glu-6-Pase activity (11–13) and failed to demonstrate any significant transport of glucose into rat liver microsomes under zero-trans uptake conditions (13, 14). Those studies have resolved, however, neither the nature of the labeled species that equilibrate during the rapid phase of intramicrosomal uptake nor the kinetics of the relationship between fast tracer uptake and Glu-6-P hydrolysis and between rapid and slow tracer rates. Since all of those issues appear critical to the validation of the substrate transport (1–7) or conformational (9–12) models, they have been addressed in the present studies. Our results demonstrate that the steady-state rate of Glu-6-P hydrolysis by intact microsomes exceeds by more than 10-fold the steady-state rate of glucose efflux from microsomes, thus ruling out the substrate transport model (1–7). It is further shown that the tracer uptake during the rapid phase of intravesicular accumulation is not the precursor for hydrolysis during the slow uptake phase but represents instead an exchangeable glucose pool. Accordingly, we propose a modified version of the combined flexibility-substrate transport model (9, 10) in which glucose released by Glu-6-P hydrolysis accumulates within a hydrophilic pocket and has access to either of the extra- or intramicrosomal compartments through outer and inner channels with different intrinsic permeabilities to glucose.

EXPERIMENTAL PROCEDURES

Microsome Purification, Uptake Measurements, and Assays—All results reported herein were obtained using the experimental conditions described in the companion paper (13) and mostly refer to a more extensive analysis of the data shown in Fig. 1 (closed symbols) of that paper.

Presentation of Data and Kinetic Analyses—Intramicrosomal concentrations have been calculated from the intravesicular content by assuming an intravesicular volume of 0.8 ± 0.1 μl/mg protein (11). The steady-state (Vss) and initial burst rates (V1) of glucose production, the latter being assimilated to the steady-state rate of Glu-6-P hydrolysis in microsomes permeabilized with 0.4% deoxycholate (Vssop) (11), were determined using Equation 1 of Ref. 13. The steady-state levels of intramicrosomal tracer accumulated during the rapid (AMP1) and slow (AMP2) phases of accumulation were estimated using Equation 2 of Ref.

* This work was supported by Grants ME-10783 and MT-10804 (to G. v. d. W.) from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Dept. of Nutrition, Université de Montréal, C.P. 6128, Succursale "centre-ville" Montréal, Québec H3C 3J7, Canada. Tel.: 514-343-7546; Fax: 514-343-6103; E-mail: vandeweg@ere.umontreal.ca.

‡ The abbreviations used are: Glu-6-Pase, glucose 6-phosphatase; Glu-6-P, glucose 6-phosphate; ER, endoplasmic reticulum; T1, liver microsomal glucose 6-phosphate translocase; T2, liver microsomal phosphate translocase; T3, liver microsomal glucose translocase; FSRFA, fast sampling, rapid filtration apparatus (U. S. patent 07/697,769).

Vol. 270, No. 36, Issue of September 8, pp. 21098–21102, 1995
Printed in U.S.A.
Steady-state concentrations of tracer accumulated into microsomes during the fast (AMP₁) and slow (AMP₂) uptake phases following incubation with varying concentrations of [¹³C]Glu-6-P

| [Glu-6-P] | [AMP₁] | [AMP₂] |
|-----------|--------|--------|
| mM       | Exp.   | Th.    | Exp.   | Th.    |
| 0.2       | 0.24 ± 0.07 | 0.23   | 2.40 ± 0.10 | 2.39   |
| 0.5       | 0.42 ± 0.12 | 0.48   | 4.85 ± 0.18 | 4.94   |
| 1         | 0.94 ± 0.22 | 0.76   | 8.03 ± 0.39 | 7.65   |
| 2         | 0.95 ± 0.36 | 1.08   | 10.37 ± 0.48 | 10.55  |
| 5         | 1.67 ± 0.68 | 1.44   | 12.91 ± 0.77 | 13.66  |
| 10        | ND     | 1.62   | 18.31 ± 1.54 | 15.14  |

Experimental values were calculated as described in the text from the data of Fig. 1 (closed symbols) of Ref. 13. Theoretical values were calculated from the kinetic parameters of Table II. Numbers under brackets represent the ratio of intramicrosomal tracer to extravesicular G6P concentrations. ND, not determinable due to the low signal over noise ratio.

The initial rates of tracer uptake during the slow phase of intramicrosomal accumulation were found to be comparable at AMP₁ and AMP₂. Moreover, the approach to a steady-state level of tracer uptake when using subsaturating Glu-6-P concentrations was observed, and in agreement with our former conclusion (11, 13) that [¹³C]glucose is the major species present within the steady-state tracer accumulation when using subsaturating Glu-6-P concentrations. Moreover, the apparent decreasing accumulation ratio observed with AMP₁ at increasing Glu-6-P concentrations is compatible with a situation where the Vₘ₅₅ for Glu-6-P hydrolysis would be higher than the Vₘ₅₅ for transport while the Kₘ₅₅ of both processes would be similar (15), in agreement with the kinetic parameters shown in Table I for DOC and Vₛₛ. We thus have investigated directly the possibility that AMP₁ might represent intramicrosomal steady-state Glu-6-P concentrations by plotting the initial rates of uptake during the slow phase of tracer accumulation (Vₛₛ) against the theoretical values of [AMP₁] shown in Table I. Indeed, the expectation here was to find a Michaelis-Menten relationship with kinetic parameters similar to those characterizing Vₛₛ. As shown in Fig. 1A, however, a linear relationship is observed over the accessible range of concentrations, thus ruling out the tracer taken up during the rapid phase as the precursor for hydrolysis during the slow phase of uptake. It should be noted that the slope value of 113.5 ± 5.3 pmol/s/mg of protein is equivalent in microsomes to a first order rate constant of 0.142 ± 0.024 s⁻¹ (t₅₀ = 4.9 ± 0.8 s).

An important observation to be made in Table I is that the AMP₁/AMP₂ ratio is quite constant over the whole range of Glu-6-P concentrations with mean values of 0.105 ± 0.018 and 0.101 ± 0.004 for experimental and theoretical values, respectively. Accordingly, we were led to consider the hypothesis that [AMP₁] might represent glucose concentrations within a third compartment located in between the extra- and intravesicular spaces in which the sugar could accumulate rapidly and reach a steady-state concentration before being able to access the intramicrosomal volume to any significant extent. Compatible with this hypothesis are the results of Table I showing that: 1) AMP₁ and Vₛₛ follow Michaelis-Menten kinetics relative to outside Glu-6-P concentrations with Kₘ₅₅ similar to that of Glu-6-P hydrolysis, and 2) the AMP₁/AMP₂ ratio determined from Table I. Quite importantly too, the results of Table I demonstrate that the Vₛₛ value represents 7.4% only of that for Vₛₛ, thus showing that the amount of glucose produced from Glu-6-P hydrolysis and liberated into the intramicrosomal compartment represents a minor fraction only of glucose directly released into the incubation medium. Alternative explanations relating to the consideration that a major fraction of our membrane vesicles might be either leaky or “inside-out” oriented can be safely ruled out since the latency of glucose dehydrogenase activity is routinely 95–100% in our vesicle preparations (13), in agreement with the former demonstration that mannose-6-phosphate is not hydrolyzed to any measurable extent during the first minute of incubation and that both uptake into microsomes and total glucose production by intact vesicles are fully inhibited by 5 mM phlorizin (11). Moreover, as discussed in the companion paper (13), such results cannot be the consequence of damage to a major fraction of the vesicles when using the FSRFA. Finally, the observation that the bulk of glucose accumulation into microsomes occurs during the steady-state phase of Glu-6-P hydrolysis cannot be the result of a slow vesicle equilibration with glucose released into the incubation medium. Indeed, by taking into account both the experimental conditions (13) and the kinetic parameters for Glu-6-P hydrolysis (Table I), it can be evaluated that the glucose concentration in the extravesicular medium never exceeds 10–16 μM at 0.2 mM outside Glu-6-P over a 2-min incubation period. In fact, then, glucose can be accumulated under such conditions up to 15–20 or 160–270-fold relative to outside Glu-6-P or glucose concentrations, respectively.

A final test of the substrate transport model (1, 7) can be proposed on the rationale that, when a steady-state level of tracer accumulation into microsomes has been reached, the rate of tracer influx into microsomes (Glu-6-P transport and hydrolysis) is equal to the rate of tracer efflux from microsomes (glucose efflux). Accordingly, Vₛₛ should be equal to the initial rate of efflux from microsomes measured at 0.2 mM glucose and 1 min. Indeed, this prediction of the substrate transport model fails in Fig. 1B where both Vₛₛ (open symbols) and the initial steady-state rate of glucose efflux (closed symbols) corresponding to the efflux data of Figs. 1B and 1C have been plotted against the theoretical values of [AMP₁] shown in Table I. No saturation could be observed over the accessible range of concentrations, and the slope values of 154 ± 4 for Vₛₛ and 112 ± 6 pmol/s/mg of protein. Vₑ for efflux are equivalent in microsomes to first order rate constant values of 0.193 ± 0.029 s⁻¹ (t₅₀ = 3.59 ±
The same vesicle preparation was used to determine the steady state tracer concentrations achieved during the fast (AMP\text{f}) and slow (AMP\text{s}) phases of uptake, as well as the steady state rates of Glu-6-P hydrolysis in both intact (V\text{s}) and DOC-treated (V\text{DOC}) microsomes. Initial rates of glucose transport during the slow uptake phase (V\text{s}), and kinetic parameters have been determined as described in the text. Y\text{max} represents either V\text{max} (nmol/s/mg of protein) or AMP\text{max} (nmol/mg of protein) for the rate and amplitude data, respectively.

|              | V\text{DOC} | V\text{s} | V\text{ss} | AMP\text{f} | AMP\text{s} |
|--------------|-------------|-----------|-----------|-------------|-------------|
| K\text{m} (mM) | 1.12 ± 0.05 | 1.37 ± 0.05 | 1.10 ± 0.18 | 1.42 ± 0.53 | 1.22 ± 0.12 |
| Y\text{max} | 5.51 ± 0.15 | 2.56 ± 0.11 | 0.19 ± 0.02 | 0.48 ± 0.34 | 13.59 ± 0.83 |

**DISCUSSION**

The demonstration that most of glucose produced from Glu-6-P hydrolysis is released directly into the extramicrosomal space strongly argues against the current version of the substrate transport model (1, 7). All of the kinetic evidence presented herein and in the companion paper (13) point out, however, to intimate relationships between glucose accumulation into microsomes and Glu-6-Pase activity, and between the rapid and slow phases of glucose uptake. Moreover, our results appear quite compatible with the hypothesis of an exchangeable glucose pool located in between the extra- and intravesicular spaces. Such a view fits nicely within the concept of the combined conformational flexibility-substrate transport model of Schulze et al. (10), an updated version of which is shown in Fig. 2 and justified below.

According to Fig. 2, Glu-6-Pase is an intrinsic membrane protein that would span the entire microsomal membrane, in agreement with the deduced sequence of the enzyme catalytic subunit that predicts up to six membrane-spanning segments in the secondary structure of the protein (16, 17). The catalytic site would be embedded to some extent within the protein interior and would form a hydrophilic pocket in an otherwise hydrophobic environment. That pocket would be accessible directly from the external medium via a hydrophilic outer channel that is not, however, freely accessible to just any molecule. In that sense, there should be some gating mechanism that might account for the unidirectionality of glucose transport and allow for both substrate and inhibitor specificities (T1-like function). Glu-6-P would be hydrolyzed within the hydrophilic pocket, and glucose released at that site could be either exported to the external medium through the outer channel or transported into the intravesicular space via the inner channel (T3-like activities).

The difference in the time scales needed to reach a steady-state rate of Glu-6-P hydrolysis and a steady-state level of glucose accumulation into microsomes can be explained in a three-compartment system like the one defined by the enzyme structure postulated in Fig. 2 by assuming that Glu-6-P transport through the outer channel, Glu-6-P hydrolysis in the hydrophilic pocket, and glucose permeation through the outer channel (Fig. 2, upper part of the cycle) are all faster than glucose transport through the inner channel. If, additionally, glucose efflux through the outer channel is slower than both Glu-6-P transport into and Glu-6-P hydrolysis within the pocket, then free glucose would accumulate into that compart-
ment until a steady state is reached. At that point, the rate of glucose exit would be equal to the slower activity of either Glu-6-P transport or Glu-6-P hydrolysis. Since similar $K_m$ for hydrolysis are observed in deoxycholate-treated membranes and, in native membranes, during the burst and the steady-state phases of glucose production (Table I), we would favor at this point the hypothesis that hydrolysis is slower than Glu-6-P transport. Alternatively, Glu-6-P access to the catalytic site and hydrolysis might be tightly coupled events (10, 11). Under these conditions, Equations 1–3 apply where $V_{SS_{max}}$ and $K_m$ refer to the kinetic parameters of Glu-6-P hydrolysis during the steady-state phase of total glucose production (Table I) while ($G_m$), ($G_o$), and $k_{D2}$ are defined in the legend to Fig. 2.

$$\frac{d(G_o)}{dt} = V_{SS} - k_{D2} [G_o] = 0 \quad (Eq. 1)$$

$$\frac{d(G_i)}{dt} = k_{D2} [G_i] = V_{SS} \quad (Eq. 2)$$

$$[G_o] = \frac{1}{k_{D2}} \frac{V_{SS_{max}}(Glu-6-P)}{K_m + [Glu-6-P]} - [AMP] \quad (Eq. 3)$$

Since a steady-state rate of Glu-6-P hydrolysis insures constant $G_m$ within the hydrophilic pocket, the glucose pool can slowly equilibrate with the intravesicular space through the inner channel. Under these conditions, Equations 4 and 5 apply where ($G_i$) and $k_{D2}$ are defined in the legend to Fig. 2.

$$\frac{d(G_i)}{dt} = k_{D2} \cdot ([G_m] - [G_o]) \quad (Eq. 4)$$

$$[G_i] = [G_m] \cdot (1 - e^{-t \cdot 1/k_m}) = [AMP_2] \cdot (1 - e^{-t \cdot 1/k_m}) \quad (Eq. 5)$$

It should be stressed that the hypotheses that were made in deriving the above equations are indeed compatible with 1) the $t_{1/2}$ values in the range of 2.5–5.0 s observed for the first exponential term in the glucose accumulation curves (13) that would characterize the filling time of the hydrophilic pocket (rate constant $k_{D2}$ in Equations 1–3) and 2) the $t_{1/2}$ values in the range of 30–50 s (13) observed for the slow phase of glucose uptake into microsomes that would characterize glucose equilibration between the hydrophilic pocket and the intramicrosomal space (rate constant $k_{D3}$ in Equations 4 and 5).

An important feature of the model depicted in Fig. 2 is illustrated by Equations 3 and 5 showing that [AMP$_1$] and [AMP$_2$] both represent [G$_m$]. Accordingly, the [AMP$_1$]/[AMP$_2$] ratio in Table I, where [AMP$_1$] and [AMP$_2$] values have been calculated relative to the intramicrosomal volume, would represent in fact the apparent fractional volume of the hydrophilic pocket relative to the intramicrosomal space, hence its constant value and independence on Glu-6-P concentrations. As shown in Table II, however, both AMP$_1$ and AMP$_2$ should follow Michaelis-Menten kinetics with $K_m$ typical of Glu-6-P hydrolysis. Indeed, the condition [AMP$_1$] = [AMP$_2$] calls for a reassessment of the plot shown in Fig. 1A, which should depict, according to Equation 4 with [$G_i$] = 0, the dependence of $V_{SS}$ on [$G_m$]. Accordingly, the $t_{1/2}$ value determined in that figure (4.9 ± 0.8 s), which should characterize $k_{D3}$, is underestimated by a factor of 10. That correction thus agrees with the determination of $k_{D3}$ from 1) the uptake time courses according to Equation 5, which is equivalent to Equation 3 in Ref. 13 ($t_{1/2}$ in the range of 30–50 s), and 2) the efflux data shown in Fig. 1B (closed symbols, $t_{1/2} = 49.5 ± 8.8$ s), where glucose permeation through the inner channel represents the overall rate-limiting step of the efflux process.

The simple scheme depicted in Fig. 2 is also compatible with the following results presented herein and in the companion paper (13): 1) the linearity of $V_{SS}$ relative to ($G_i$) with slope $k_{D2}$ (Equation 2 and Fig. 1B, open symbols), and its michaelian dependence on (Glu-6-P)$_o$ (Equations 2 and 3 and Table II); 2) the independence of the $t_{1/2}$ of glucose equilibration into microsomes from the presence of varying concentrations of Glu-6-P or vanadate (13); 3) the similar steady-state levels of intramicrosomal glucose achieved under symmetrical influx and efflux conditions of Glu-6-P and vanadate concentrations (13); and 4) the direct correlation between the steady-state levels of intramicrosomal [14C]$\_\text{glucose}$ accumulation and $V_{SS}$ measured at increasing cold Glu-6-P or vanadate concentrations (13). In that context, our recent studies with histone IIA-treated microsomes (18) also agree with the model of Fig. 2 in that higher uptake values were associated with higher Glu-6-Pase activities in treated as compared with normal microsomes. Moreover, the failure of histone IIA-treated microsomes to take up [14C]$\_\text{mannose}$ from [14C]$\_\text{mannose}$ 6-phosphate (18) would suggest that the inner channel is quite specific for glucose and that histones mostly affect the gating mechanism of the upper channel.

While the molecular arrangement within the transverse plane of the microsomal membrane proposed in Fig. 2 for Glu-6-Pase does not exclude that the catalytic subunit be associated with one or several proteins (subunits), it is quite clear too that our studies (Refs. 11–13 and 19 and this paper) failed to demonstrate the existence of both T1 and T3 (GLUT7) as entities fully separated from the catalytic process. Accordingly, the scheme of Fig. 2 should be viewed as the minimum mechanistic model that is needed to explain the steady-state kinetics of Glu-6-P hydrolysis and glucose exchange through the ER membrane. That scheme, in its general principle at least, thus represents by now the only alternative to the more classic substrate transport hypothesis. The recent demonstration that the Glu-6-Pase gene of glycogen storage disease type 1b and 1c patients is normal (20) clearly indicates, however, that a major component of the Glu-6-Pase system is presently missing in the model of Fig. 2. It is thus reasonable to propose that an extra protein component(s) might be needed to account for other characteristics of the complete system, an obvious candidate(s) being a soluble or a membrane protein(s) that would modulate the transport functions through the outer and/or inner channels. Indeed, the characterization of such a component(s) should prove of paramount importance to the understanding of a number of questions that are not readily answered by the scheme of Fig. 2 like the apparent unidirectionality of glucose transport (13) and the molecular mechanism responsible for the hysteretic transition from a "detergent-like" to a "steady-state" form of the enzyme (11). Independent of the complexity of the Glu-6-Pase system, however, the model of Fig. 2 strongly suggests that the release of phosphate inside of the hydrophilic pocket might contribute to the hysteretic transition by a mechanism that has yet to be determined.

Acknowledgments—We thank C. Gauthier for the art work.

REFERENCES
1. Burchell, A. (1990) FASEB J. 4, 2978–2988
2. Nordlie, R. C. (1982) Methods Enzymol. 87, 319–353
3. Arion, W. J., Lange, A. J., Walls, H. E., and Ballas, L. M. (1980) J. Biol. Chem. 255, 10396–10400
4. Waddell, I. D., Zomerschoe, A. G., Voice, M. W., and Burchell, A. (1992) Biochem. Soc. Trans. 20, 10396–10400
5. Burchell, A. (1994) Biochem. J. 286, 173–177
Membrane Glucose Pool and Glucose-6-phosphatase Structure

9. Schulze, H.-U., and Speth, M. (1980) Eur. J. Biochem. **105**, 505–514
10. Schulze, H.-U., Nolte, B., and Kannler, R. (1988) J. Biol. Chem. **263**, 16571–16578
11. Berteloot, A., Vidal, H., and van de Werve, G. (1991) J. Biol. Chem. **266**, 5497–5507
12. Vidal, H., Berteloot, A., Larue, M.-J., St-Denis, J.-F., and van de Werve, G. (1992) FEBS Lett. **302**, 197–200
13. St-Denis, J.-F., Berteloot, A., Vidal, H., Annabi, B., and van de Werve, G. (1995) J. Biol. Chem. **270**, 21092–21097
14. Romanelli, A., St-Denis, J.-F., Vidal, H., Tchu, S., and van de Werve, G. (1994) Biochem. Biophys. Res. Commun. **200**, 1491–1497
15. Wohlueter, R. M., and Plagemann, P. G. W. (1980) Int. Rev. Cytol. **64**, 171–240
16. Lei, K.-J., Shelly, L. L., Pan, C.-J., Sidbury, J. B., and Chou, J. Y. (1993) Science **262**, 580–583
17. Shelly, L. L., Lei, K.-J., Pan, C.-J., Sakata, S. F., Ruppert, S., Schutz, G., and Chou, J. Y. (1993) J. Biol. Chem. **268**, 21482–21485
18. St-Denis, J.-F., Annabi, B., Khoury, H., and van de Werve, G. (1995) Biochem. J. **310**, 221–224
19. St-Denis, J.-F., Comte, B., Nguyen, D. K., Seidman, E., Paradis, K., Levy, E., and van de Werve, G. (1994) J. Clin. Endocrinol. Metab. **79**, 955–959
20. Lei, K.-J., Shelly, L. L., Lin, B., Sidbury, J. B., Chen, T.-T., Nordlie, R. C., and Chou, J. Y. (1995) J. Clin. Invest. **95**, 234–240