Ontogeny and plasma-membrane domain localization of amino acid transport system L in rat liver*

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Na⁺-independent hepatic transport of branched-chain amino acids occurs via at least two distinct transport processes. System L₁, characterized by micromolar K_m values, predominates in hepatoma and fetal hepatocytes, whereas System L₂, distinguished by K_m values in the millimolar range and sensitivity to inhibition by *N*-ethylmaleimide (NEM), predominates in adult hepatocytes. To determine the plasma-membrane domain localization and ontogeny of System L activity in the rat, we prepared membrane vesicles from the livers of suckling (10 days old) and adult rats enriched for either basolateral (BLMV) or canalicular (CMV) domains. The initial rate of [³H]leucine uptake into BLMV and CMV derived from adult liver was significantly inhibited by the addition of 5 mM NEM; transport into BLMV and CMV derived from 10-day-old rat liver was not affected.

Michaelis–Menten kinetic parameters estimated in BLMV derived from adult liver were consistent with System L₂ ($K_m = 2.16 \pm 0.62$ mM, $V_{max} = 781 \pm 109$ pmol/5 s per mg of protein), as were those estimated in adult CMV ($K_m = 0.83 \pm 0.21$ mM, $V_{max} = 385 \pm 38$ pmol/5 s per mg of protein). Conversely, kinetic parameters estimated in BLMV derived from livers of suckling rats were consistent with System L₁ ($K_m = 0.041 \pm 0.024$ mM, $V_{max} = 8.8 \pm 1.5$ pmol/5 s per mg of protein), as were those from CMV of suckling rats ($K_m = 0.023 \pm 0.09$ mM, $V_{max} = 28.1 \pm 2.1$ pmol/5 s per mg of protein). We conclude that NEM-inhibitable Na⁺-independent leucine transport activity consistent with System L₂ is present in both BLMV and CMV derived from adult rat liver, whereas System L₁ predominates in 10-day-old rat liver tissue.

INTRODUCTION

The newborn grows at a pace unmatched throughout later life, generally doubling its birth weight by 5 months of age. Amino acids serve as substrates for the production of proteins and as precursors of important metabolites such as glucose and neurotransmitters [1]. The liver, as a central site of amino acid metabolism, plays a critical role during periods of rapid growth. Transport of amino acids into the hepatocyte from the portal circulation can be the rate-limiting step in amino acid metabolism [2]. Additionally, excretion of amino acids across the hepatocyte canalicular membrane into the biliary lumen is thought to be important in the generation of bile flow [3], particularly during the neonatal period, when rates of both bile flow and bile acid excretion are diminished as compared with the adult [4,5].

Amino acid transport System L is responsible for the Na⁺-independent transport of neutral amino acids, particularly those with bulky branched side chains [6]. This activity, associated with Na⁺-independent transport of most of the 'essential amino acids', appears to be ubiquitous in its distribution. Transport activity identified with System L has been noted in freshly isolated hepatocytes [7], but studies in monolayers of cultured hepatocytes suggested the presence of two components of System L transport, L₁ and L₂ [8–10]. System L₂ is a low-affinity system (K_m values in the millimolar range), whereas L₁ is a high-affinity system (K_m values in the micromolar range). System L₂ predominates in freshly isolated adult hepatocytes, accounting for approx. 90% of leucine uptake. Conversely, those studies demonstrated that System L₁ predominates in hepatocytes from fetal and suckling rats as well as in transformed cells [9]. System L₂ activity declines and System L₁ becomes increasingly prominent in adult-rat

hepatocytes maintained in cell culture for 24 h or longer [10]. These data imply differential, although as yet unexplored, functions for Systems L₁ and L₂ in rapidly dividing versus quiescent cells, and suggest an enhanced role for System L₁ in fetal/neonatal life. However, the distribution of Systems L₁ and L₂ between the basolateral and canalicular membranes of the hepatocyte has not been previously explored. Precedent clearly exists for sub-domain localization of amino acid transport systems [11,12]. It is likely that membrane domain distribution is important with regard to transporter function. For these reasons, and to understand better the changes in transporter function occurring during cell culture, we have chosen to study the distribution of Systems L₁ and L₂ in canalicular and basolateral enriched plasma-membrane vesicles derived from 10-day-old and adult rat liver.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats, 10 days old (8–12 pups per litter) or adult (200–250 g) were obtained from Harlan Laboratories (Indianapolis, IN, USA). The rats were housed in a temperature-controlled room with 12 h light/dark cycles. Adult rats were given free access to standard rat chow and water, while pups were still suckling.

Preparation of rat liver canalicular and basolateral membrane vesicles (CMV and BLMV)

BLMV were prepared by the method of Blitzer and Donovan [13], with modifications as described by Suchy and co-workers

Figure 1 Leucine uptake by rat liver BLMV and CMV

The uptake of $0.12 \mu\text{M}$ [^3H]leucine into adult BLMV (ABLMV), adult CMV (ACMV), suckling-rat BLMV (SBLMV) and suckling-rat CMV (SCMV) in the absence (●) and presence (■) of 10 mM unlabelled leucine was measured at 30°C as described in the Materials and methods section. Data are expressed as means $\pm$ S.D. Where not shown, the S.D. bars are contained within the symbol.

[14]. CMV were prepared by a modification of the method of Inoue et al. [15], as previously described by Novak et al. [16]. Membrane samples were rapidly frozen and stored in liquid N_2 until used. Protein concentrations were determined by the method of Lowry et al. [17], with BSA as standard. The membrane preparations utilized have previously been characterized in this laboratory, as well as by others, and found to be enriched in the appropriate membrane fraction with minimal contamination [13–16,18]. Both preparations have comparable marker enzyme profiles and volumes in the age groups studied [14,16]. They are therefore well suited for these developmental studies.

Transport assays

The uptake of [^3H]leucine (New England Nuclear, Boston, MA, U.S.A.) was determined as previously described [16]. In brief, $20 \mu\text{l}$ portions of membrane vesicles (approx. $50 \mu\text{g}$ of protein) suspended in 0.3 M sucrose containing 10 mM Hepes/KOH buffer, pH 7.5, were preincubated in a glass culture tube at 30°C for 2–4 min. Uptake studies were then initiated by addition of $80 \mu\text{l}$ of incubation solution (10 mM Hepes/KOH, 125 mM KCl, 0.25 mM CaCl_2 , 12.5 mM MgCl_2 , pH 7.5, indicated concentrations of radioactively labelled substrate, with additional sucrose to maintain osmolarity at 310 mosM) to the preincubated membrane suspension. Timed uptakes were terminated by rapid addition of 3 ml of ice-cold stop solution (100 mM KCl, 100 mM sucrose, 10 mM Hepes/KOH, pH 7.5) and filtered through a $0.45 \mu\text{m}$ -pore nitrocellulose filter pre-wetted with 3 ml of 1 mM leucine to decrease non-specific adherence of substrate. The tube was rinsed with an additional 3 ml of ice-cold stop solution, which was then filtered. The filter was washed further with $2 \times 3 \text{ ml}$ of stop solution, placed into a vial, dissolved in 4.5 ml of scintillation fluid (ScintiSafe; Fisher Scientific, Pittsburgh, PA, U.S.A.) and the radioactivity (d.p.m.) was determined in a scintillation spectrophotometer. Uptakes were corrected with a 'blank' in which 3 ml of ice-cold stop solution was added to the

membrane sample before addition of radiolabelled leucine. This blank value was subtracted from each experimental uptake value. Transport measured in the presence of *N*-ethylmaleimide (NEM) was performed after a 15 min incubation of membrane with 5 mM NEM at room temperature. Initial rates of transport were estimated from uptakes at 5 s in the absence or presence of 5 mM NEM. All incubations were performed at least in triplicate.

Data analysis

Kinetic analysis was performed by using computer-assisted least-squares fit of data points to rectangular hyperbolas [19]. Diffusion constants were directly estimated in adult-rat membrane preparations as the difference between the uptake of 10 mM leucine in the presence and absence of 10 mM NEM. The K_a derived in this way was $110 \text{ nM}/5 \text{ s}$ per mg of protein for both CMV and BLMV. Conversely, because uptake in vesicles derived from suckling-rat liver was not NEM-inhibitable, a computer-assisted model was utilized for hepatic membrane vesicles derived from 10-day-old animals. K_a values derived were 283 (CMV) and $(\text{BLMV}) 289 \text{ nM}/5 \text{ s}$ per mg of protein. Data from all preparations were examined for the presence of multiple transport systems both graphically and with the aid of a computer. Differences between groups were determined by using Student's two-tailed *t* test.

RESULTS

To perform kinetic analysis of substrate uptake, the rate of uptake must be linear with regard to the time over which transport is assayed. The uptake of 2 mM leucine was linear until approx. 12 s (results not shown). To ensure linearity, a 5 s uptake point was chosen for all of the data reported. Figure 1 details the early phase of the time course for uptake of [^3H]leucine into either BLMV or CMV derived from adult and 10-day-old animals. Equilibrium uptake values in CMV were approximately

Table 1 Inhibition of leucine uptake by NEM

Vesicles were preincubated with 5 mM NEM for 15 min at 25 °C. The uptake of 10 mM [3 H]leucine was then measured for 10 s at 30 °C. The numbers shown include both saturable and non-saturable uptake. The data are expressed as pmol/10 s per mg of protein and are means $\pm$ S.E.M.: * $P \leq 0.02$ compared with control adult BLMV; ** $P \leq 0.005$ compared with control adult CMV.

Vesicle preparation	Control	NEM
Adult BLMV	2158 $\pm$ 212	1022 $\pm$ 213*
Adult CMV	1729 $\pm$ 282	620 $\pm$ 200**
Suckling BLMV	2334 $\pm$ 326	1789 $\pm$ 349
Suckling CMV	2419 $\pm$ 342	2260 $\pm$ 480

twice those in BLMV (results not shown), in agreement with previously defined differences in the respective vesicle volumes [14,16]. In all cases, uptake of 0.12 μ M [3 H]leucine was markedly inhibited by addition of 10 mM leucine, demonstrating the saturable nature of the transport (Figure 1). Table 1 illustrates the inhibition by 5 mM NEM of 10 mM leucine uptake in adult BLMV and CMV, but not in vesicles derived from 10-day-old animals. NEM, a thiol reagent which is able to penetrate cell membranes and, via reaction with exposed cysteine residues, to inhibit a variety of cell-membrane activities, has previously been shown to inhibit System L₂ without non-specific effects on hepatocyte membrane permeability [20]. Figure 2 details the kinetic analysis of leucine uptake by BLMV and CMV from both adult and 10-day-old animals. In accordance with the NEM-inhibition data, kinetic constants obtained in membrane vesicles derived from adult animals were consistent with those associated with System L₂ activity, whereas those obtained with vesicles from 10-day-old animals were similar to previously reported values for System L₁. Thus a System L-mediated amino acid

transport activity appears to be present on both hepatocyte membrane surfaces at both stages of development. Consistent with earlier studies [9], System L₁ activity is the primary mode of transport in younger animals, whereas its contribution declines with age and the expression of System L₂ activity is increased such that it represents the primary transporter in the adult tissue.

DISCUSSION

Na⁺-independent leucine uptake into the hepatocyte has been shown to occur via two transport systems, L₁ and L₂ [8–10]. The present study uses isolated membrane vesicles to extend previous observations which were made with isolated rat hepatocytes. The low-affinity Na⁺-independent amino acid transport System L₂ appears to play a predominant role in adult liver, whereas the higher-affinity but lower-capacity System L₁ predominates in the livers and suckling rats. Although previous work [10] suggested the presence of System L₁ activity in cultured hepatocytes derived from adult rats, we were unable to detect System L₁ in this age group, despite determination of leucine uptake at multiple concentrations within the expected range of this system (0.5–100 μ M). The presence of System L₁ in the previous work may have reflected the influence of cell culture, because it was demonstrated that the expression of System L₁ is low in freshly isolated hepatocytes, comparable with that in isolated vesicles, and then increases with time in culture [9,10].

We also have demonstrated, for the first time, the domain localization of System L transport in the hepatocyte plasma membrane. As estimated from apparent rate constants ($V_{\max}/K_m$), transport rates mediated by System L are highest across the canalicular membrane for both age groups, despite the fact that System L₁ is mediating this transport in the younger animals and System L₂ activity is responsible for the transport in the adult. The difference between the rates in the basolateral and canalicular domains was much greater in suckling animals. We have utilized the first-order rate constant ($V_{\max}/K_m$) as a means

Figure 2 Kinetic analysis of leucine uptake into rat liver BLMV and CMV

The transport of [3 H]leucine (0.0005–10 mM) into adult BLMV (ABLMV), adult CMV (ACMV), suckling-rat BLMV (SBLMV) or suckling-rat CMV (SCMV) was measured for 5 s at 30 °C, and the kinetic constants were determined as outlined in the Materials and methods section. Plots shown are computer-derived best fits after subtraction of the non-saturable uptake. The apparent rate constants were estimated as the $V_{\max}/K_m$ ratio. K_m values are given in mM, and $V_{\max}$ values are in pmol/5 s per mg of protein.

to emphasize the importance of both substrate affinity and translocation velocity when comparing net substrate transport across distinct membranes, although we recognize the limitations of this constant, particularly when substrate concentrations approach K_m values. The presence of System L on the canalicular membrane is not surprising, given the relatively high branched-chain amino acid concentrations noted in bile [21]. Proton gradients are thought to stimulate System-L-mediated transport [22]. Leucine efflux from the hepatocyte via System L may therefore be stimulated by the lower pH of the hepatocyte cytoplasm as compared with the interior of the bile canaliculus [23]. The excretion of amino acids into bile may have a choleric effect [3]; the presence of an efficient amino acid transport mechanism in the neonatal-rat canalicular membrane might help to counteract the diminished rates of bile acid secretion noted in this age group [4,5].

Fetal- and neonatal-rat hepatocyte basolateral membranes are exposed to relatively high portal venous amino acid concentrations as compared with those found in the adult [24]. Leucine flux into the adult liver from the portal-venous system is negligible [25]. The lower V_{max}/K_m value noted on the neonatal-rat basolateral-membrane surface may serve to limit the influx of leucine into the developing liver, and thus maintain hepatic leucine concentrations similar to or lower than those noted in the adult liver [26,27] in the face of significantly higher serum leucine concentrations [24,27]. Limitation of the influx of System L substrates, many of which are essential amino acids, into the liver might then increase their availability to developing extrahepatic tissues [28–30].

Collectively, the results in the present paper demonstrate that the relative rate of System-L-mediated transport across the hepatocyte basolateral and canalicular membranes is not a function of differential expression of Systems L₁ and L₂ between these surfaces. In fact, despite a developmentally regulated shift in predominance from System L₁ to System L₂, total leucine transport across the canalicular membrane domain remains more rapid than that across the basolateral surface.

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