Molecular and functional analysis of glutamine uptake in human hepatoma and liver-derived cells

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WHETHER OBSERVED CLINICALLY or experimentally, the transformation of cells to a cancerous phenotype is often associated with cognate changes in the transport and metabolism of nutrients such as glucose and glutamine (4, 37). Glutamine serves as the primary ammonia shuttle between tissues and as a major carbon and nitrogen source for many tumors (39), whereas the liver serves as the major regulatory center for whole body ammonia detoxification and glutamine homeostasis (26, 40). This regulatory role is subverted on hepatocellular transformation when glutamine consumption often prevails (8), an observation underscored clinically by lower plasma levels of this amino acid in liver cancer patients (29). Similarly, depressed glutamine synthetase rates with concomitantly augmented glutamine oxidation and glutaminase rates have been reported in human hepatomas compared with normal livers (9, 36, 37). Indeed, the observation that many tumor cells exhibit enhanced rates of glutamine metabolism has led to the testing of glutamine analogs as anticancer agents (2).

It has been recognized that the transport of glutamine across the plasma membrane of liver cells may represent a rate-limiting step in its metabolism, especially when intracellular catabolism is accelerated (27, 33). In normal rat and human hepatocytes, glutamine transport is predominantly mediated by an Na+-dependent transporter with narrow substrate specificity (glutamine, histidine, and asparagine) termed system N (6, 32). Two separate genes encoding for this activity, termed SN1 and SN2, have recently been isolated (19, 42). Previously, the initial-rate transport of glutamine in three human hepatoma cell lines (SK-Hep, HepG2, and Huh-7) was functionally examined and found to exceed rates in normal human hepatocytes by 10- to 30-fold, due to the expression of a distinct higher affinity Na+-dependent carrier with characteristics of system ASC (6, 9). A cDNA was subsequently isolated that encodes for a broad specificity Na+-dependent glutamine transporter, termed amino acid transporter B⁰ (ATB⁰) (30, 31), whose properties match those previously reported for the system ASC-like hepatoma activity. Recent studies suggested that this system ASC activity may govern glutamine-dependent growth in the SK-Hep human hepatoma cell line (7). On the basis of these results, the hypothesis evolved that system ASC expression may be an integral event in human hepatocellular transformation. The studies presented here were therefore designed to determine the ubiquity of system ASC-mediated glutamine transport in prolif-
erating liver cells, to identify the gene responsible for this activity, and to determine its utility in hepatocellular growth.

MATERIALS AND METHODS

Cell Lines. The human hepatoma cell lines used in these studies were PLC/PRF/5 (34), SK-Hep (20), Hep3B (ATCC, Rockville, MD) (1), HuH-7 [from Dr. J. Liang; Massachusetts General Hospital (MGH)] (41), FOCUS (from Molecular Hepatology Laboratories, MGH Cancer Center) (28), and the hepatoblastoma HepG2 (1), also from ATCC. A nontumorigenic human liver epithelial cell line termed THLE-5B was generated by immortalization with SV40 virus and was kindly provided by Dr. C. Harris at the National Cancer Institute (45). The JAR-1 human choriocarcinoma cell line, from which the ATB<sup>D</sup> cDNA was originally isolated, was obtained from ATCC. All cells were maintained in DMEM (4.5 mg/ml d-glucose) + 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 10% FBS (all from Gibco-BRL, Gaithersburg, MD).

Human liver tissue. The normal, cirrhotic, and cancerous human liver tissue used for RNA analysis in these studies was from archived cryopreserved biopsies previously obtained from patients undergoing gastrointestinal surgery, as per the guidelines approved by the MGH Human Studies Committee and Institutional Review Board. Primary human hepatocytes were isolated as previously described in detail from freshly discarded pathological specimens (6). Primary human adult and fetal liver fibroblasts were isolated from primary cultures of liver cells after maintenance in the presence of 10% FBS. After 7–10 days, the fibroblasts overgrew the culture and were serially passaged by trypsinization. The fibroblasts were used between passages 3 and 10 in these studies. The primary hepatoblastoma sample was obtained from the MGH tumor bank. The fetal human liver RNA blot was kindly provided by Dr. D. Rhoads. A commercially available human liver cancer RNA blot (Northern Territory) was kindly provided by Dr. D. Rhoads. A commercially available human liver cancer RNA blot (Northern Territory) was kindly provided by Dr. D. Rhoads. A commercially available human liver cancer RNA blot (Northern Territory) was kindly provided by Dr. D. Rhoads.

Glutamine transport assays. Measurement of initial-rate glutamine uptake was carried out via the cluster-tray method originally described by Gazzola et al. (21) as reported previously (6, 9). Briefly, after trypsinization, hepatoma cells were plated at a density of 1 × 10<sup>5</sup> cells/well in 24-well culture plates (Costar, Cambridge, MA) and allowed to grow to ~80% confluence, typically 1–2 days later. For initial-rate measurements, the radiotracer used was L-[G-<sup>3</sup>H]glutamine (Amersham, Arlington Heights, IL) at 4 μCi/ml in the presence of unlabeled L-glutamine at 50 μM. For kinetic studies, the amount of unlabeled glutamine in the transport buffer varied from 10 μM to 10 mM. All transport measurements were carried out at 37°C and were terminated after 30 s by three rapid washes with an ice-cold phosphate-buffered saline solution. Intracellular radiolabeled glutamine was extracted with 0.2 ml/well of 0.2% SDS and 0.2 N NaOH; after 1 h, 0.1 ml of the lysate was neutralized with 10 μl 2 N HCl and subjected to scintillation spectrophotometry in a Packard TopCount (Packard Instruments, Meriden, CT). The remaining lysate was used for the determination of cellular protein by the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Rates of glutamine transport were calculated from the counts per minute (cpm) per sample, the specific activity of the uptake mix (in cpm/nmol), and normalized to cellular protein content in a Microsoft Excel spreadsheet program. Transport values obtained in the absence of extracellular Na<sup>+</sup> (diffusion and Na<sup>+</sup>-independent uptake) were subtracted from those in the presence of Na<sup>+</sup> (total uptake) to yield Na<sup>+</sup>-dependent rates that are reported in units of nanomoles per milligram protein per 30 s. All transport values depicted are the average ± SD of four separate determinations. Kinetic analysis of Eadie-Hofstee linearized transport data (V vs. V/[L-GLN]) was performed by regression analysis (Cricket Graph, Computer Associates, Islandia, NY). Nonlinear regression analysis was performed with DataDesk (Data Description, Ithaca, NY) and Excel for two component Michaelis-Menten kinetics: V = (V<sub>max2</sub> × S)/(K<sub>m2</sub> + S), where V is velocity, V<sub>max</sub> is maximum velocity, and S is glutamine concentration (in mM).

Glutamine-dependent growth. Cells were plated in 96-well tissue culture plates (Falcon Labware, Franklin Lakes, NJ) at a density of 2 × 10<sup>3</sup> cells/cm<sup>2</sup>. The following day, the cells were rinsed once and repleted with DMEM + 10% dialyzed FBS (dFBS) + 0, 0.05, 0.10, 0.15, 0.2, 0.4, 0.6, 0.8, 1.0, or 2.0 mM L-glutamine, with media changes every 48 h thereafter. In a subset of experiments designed to test the role of system ASC/B<sup>D</sup>-mediated glutamine uptake in cellular proliferation, cells were grown in DMEM + 10% dFBS containing 0, 0.5, or 2.0 mM L-glutamine ± the ASC substrate alanine, serine, and threonine (6.7 mM each). At specific times over 7 days, the relative cell number per well was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide colorimetric assay (Sigma, St. Louis, MO) on a plattereadere (Anthos Labtec, Frederick, MD) at a wavelength of 550 nm with a 650-nm reference filter. Relative growth rates were evaluated graphically with optical density as a function of time (days).

RNA isolation and Northern blot procedure. Total cellular RNA was isolated from cultured cells or frozen tissue by the one-step acid-phenol guanidinium procedure (13) using Trisolv reagent (Biotex, Houston, TX), subsequent treatment with RQ1 RNase-free DNase-I (Promega, Madison, WI), followed by an additional acid-phenol, phenol/chloroform/isoamyl alcohol, chloroform extraction, and ethanol precipitation in the presence of sodium acetate. Equal amounts of total RNA (20 μg), as determined both spectrophotometrically and through ethidium bromide staining, were fractionated by electrophoresis through denaturing 1% agarose gels containing 0.2 M formaldehyde, transferred to nylon membranes by capillary action and ultraviolet cross-linked to the membrane.

The DNA templates used in this study were full-length human ATB<sup>D</sup> [hATB<sup>D</sup>, 2.9-kb EcoRI insert (30)], human SN1, 2.4-kb insert (19); and human AT2A, 4.5-kb insert (25); all in pSPORT1 and kindly provided by Dr. V. Ganapathy. Human albumin (pMLALB, 1.8-kb EcoRI/Hind III fragment; α-fetoprotein (AFP; pHAF7), 492-bp Pst I fragment; IGF-II (phins311), 8.6-kb EcoRI genomic DNA insert; and glutamine synthetase (GS) HIBBA40, 2.7-kb Hind III/Not I insert) were also used and were all obtained from ATCC. The inserts containing primarily coding sequence were excised from the plasmids with appropriate restriction enzymes, separated on agarose gels, excised, eluted, and used as templates to generate α-[<sup>32</sup>P]dCTP-labeled probes using a random primer labeling kit (Megaprime, Amersham) according to the manufacturer’s protocol. Hybridization with random hexamer-generated radiolabeled DNA probes was performed overnight at 65°C in 5× sodium chloride-sodium phosphate-EDTA buffer (SNE) with 5× Denhardt’s reagent + 0.5% SDS and 0.1 mg/ml sheared herring sperm DNA, after incubating the membrane for 2 h under the same conditions.

Two of the DNA templates used in this study, human SN2 (42) and human AT3A (23), were generated by RT-PCR from human liver RNA and engineered to contain SP6 sites.
(5’AATTTAGGTCACACTAGAG3’) in the 3’ termini for generation of riboprobe as described below. A 302-bp human SN2 cDNA representing bases 3–304 of the coding sequence was generated using a sense primer (5’GGAACTGCAAGGATCGAAG3’) and antisense primer (5’AGGTACGCAGGAGTTGGATG3’). Likewise, a 303-bp human ATA3 cDNA representing bases 1–303 of the coding sequence was generated using sense 5’ATGAGCTCCATGGAACTGAG3’ and antisense (5’GCCCAGGATAGGGCAAGG3’) primers. Products of the expected size were obtained and verified by restriction endonuclease analysis. Riboprobes from PCR-generated and full-length cDNAs were generated using the Maxiscript SP6 kit from Ambion (Austin, TX) and α-[32P]UTP (Amer sham). Ultra-Hyb (Ambion) at 72°C was used in blots hybridized with riboprobe.

In all experiments, blots were washed under high stringency conditions (0.1× SSPE + 0.5% SDS at hybridization temperature), and autoradiographic detection of the hybridization was achieved by exposure of Fuji Medical X-ray film at −80°C. In some experiments, the hybridized probe was stripped off the membrane by boiling in 0.1% SDS and the blots reutilized for the Northern analyses of other genes. Where indicated, band intensities were quantified using the Kodak EDAS 290 system with one-dimensional image analysis software (Eastman Kodak, New Haven, CT).

RT-PCR and restriction enzyme analyses. ATB0 expression in individual cells and tissues was confirmed by RT-PCR and subsequent digestion with Sal I or Rsa I as described by Kekuda et al. (31). The Perkin-Elmer GeneAmp kit was used according to the manufacturer’s instructions. The upstream primer was 5’CCGCTGATGATGAAGTCG3’, and the downstream primer was 5’CCCAGGATAGGGCAAGG3’, which encompass nucleotides 1691–2197 of the hATB0 cDNA, yielding an expected amplification product of 507 bp. The RT-PCR reaction was carried out on RNA samples previously treated with RQ1 RNase-free DNase according to the manufacturer’s instructions (Promega). RNA (1 μg) was primed with oligo(dT), reverse transcribed, and subjected to 25 rounds of amplification (94°C/30 s, 60°C/30 s, 68°C/30 s for denaturation, annealing, and extension steps, respectively). The RT-PCR products were isolated from the reaction by QiAquick PCR purification kits (Qia gen, Santa Clarita, CA) before analytical endonuclease digestion. The expected Sal I digestion products were 277, 191, and 39 bp, and those for Rsa I are 330, 114, and 63 bp. All restriction enzymes were from Promega. The absence of RT in the reaction served as a negative control for all samples.

Statistical analysis. Differences in specific measured parameters were evaluated for statistical significance by paired t-test (Microsoft Excel) and were considered significant when P < 0.050.

RESULTS

Glutamine transport. In all cell lines examined, 90% or greater of glutamine uptake was Na+ dependent: Hep3B, 90%; FOCUS, 92%; PLC/PRF/5, 95%; THLE-5B, 99%. Initial rate Na+-dependent transport velocities for 50 μM L-glutamine was determined in the three additional human hepatoma cell lines, and the results are shown in Fig. 1. Markedly enhanced rates (0.77–1.01 nmol·mg protein−1·30 s−1) were again observed compared with values for normal adult and fetal human hepatocytes [0.075–0.15 and 0.36–0.44 nmol·mg protein−1·30 s−1, respectively (6)]. Transport velocities were higher (P < 0.010) in the non-tumorigenic 5V40-immortalized human liver epithelial THLE-5B cell line.
(3.33 ± 0.16) vs. the FOCUS (0.83 ± 0.13), Hep3B (1.01 ± 0.22), and PLC/PRF/5 (0.77 ± 0.10 nmol-mg protein\(^{-1}\)·30 s\(^{-1}\)). The glutamine concentration used in these assays not only allowed the measurement of initial-rate transport values but also tested the ability of each line to take up low extracellular levels of this amino acid. When the transport of glutamine at physiological levels (500 μM) was measured in each of the lines, the results were qualitatively similar (data not shown).

Figure 1 also shows that the expression of a system ASC-like transporter activity was largely responsible for the accelerated glutamine uptake in the additional four human cell lines, consistent with previous results obtained in the HepG2, SK-Hep, and Huh-7 cell lines (6, 9). This conclusion is based on significant (>80–90%) inhibition by alanine, serine, and cysteine relative to all other amino acids tested, including the system A-specific substrate α-(methylamino)isobutyric acid (MeAIB), which failed to significantly inhibit glutamine uptake in all cell lines except the THLE-5B, in which it diminished uptake by 29% \((P < 0.050)\). It should be noted that extensive depletion of intracellular amino acid pools was not performed before transport measurements in these studies, because we sought to assess glutamine uptake under more physiological conditions. As a result, low-affinity transinhibitable systems, such as system A, may not be operative or detectable under these conditions even when expressed (see molecular analyses below).

Subsequent molecular studies (described in Northern analysis of GS and other glutamine transporters) revealed that more than one potential glutamine transporter was expressed in most cell lines however. Therefore, two-component nonlinear regression analyses were performed on the Na\(^+\)-dependent transport kinetic data, where they were adequately resolved into high- and low-affinity components in all cell lines under study. The results are graphically depicted in Fig. 1, insets. The Hep3B low-affinity system exhibited a calculated \(K_m\) of 1.0 mM and \(V_{max}\) of 4.5 nmol-mg protein\(^{-1}\)·30 s\(^{-1}\), whereas the high-affinity system had a derived \(K_m\) of 90 μM and a \(V_{max}\) of 3.0 nmol-mg protein\(^{-1}\)·30 s\(^{-1}\) (Fig. 1B). The PLC/PRF/5 low-affinity system was determined to possess a \(K_m\) of 775 μM and \(V_{max}\) of 3.9 nmol-mg \(^{-1}\) protein·30 s\(^{-1}\), while the high affinity system exhibited a derived \(K_m\) of 23 μM and a \(V_{max}\) of 0.82 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) (Fig. 1D). For FOCUS, the low-affinity system had a \(K_m\) of 1.2 mM and a \(V_{max}\) of 2.7 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\), and the high-affinity component possessed a \(K_m\) of 122 μM with a \(V_{max}\) of 2.2 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) (Fig. 1C). For THLE-5B, the low-affinity component exhibited a \(K_m\) of 1.3 mM and a \(V_{max}\) of 32.6 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\), whereas the high-affinity component had a \(K_m\) of 135 μM and a \(V_{max}\) of 30.8 nmol·mg protein\(^{-1}\)·30 s\(^{-1}\) (Fig. 1A). Previous studies showed that the Huh-7 cell line took up glutamine primarily by system ASC based on amino acid inhibition profiles (9), so those results are not shown again. However, the kinetic analysis here revealed two resolvable components in this cell line with \(K_m\)s of 50 and 861 μM and \(V_{max}\) values of 7.3 and 11.7 nmol-mg protein\(^{-1}\)·30 s\(^{-1}\) for the high- and low-affinity components, respectively. The high-affinity component in all cases is assumed to be system ASC based on the amino acid inhibition data and previously reported kinetic constants for this transporter (6, 9, 14, 15, 30, 31), whereas the low-affinity components are more difficult to distinguish given that the system A and N isoforms isolated and characterized to date all possess similar affinities for glutamine in the high micromolar and low millimolar ranges (19, 23, 25, 42). Possibilities for each cell line will be offered discussion based on the molecular analyses described below.

Finally, on the basis of the derived kinetic constants listed above, it was calculated that the high-affinity component (system ASC) mediates >80% of glutamine uptake at initial-rate concentrations (50 μM) and 60–70% of uptake at physiological concentrations (600 μM) for all of the cell lines under study.

Northern blot analysis of ATB\(^0\). A cDNA encoding for a transporter with nearly identical characteristics as the system ASC activity described here was previously isolated from a human placental choriocarcinoma library (30). This gene was termed ATB\(^0\), for the transport activity designated as system B\(^0\), originally described in intestinal epithelial cells (49) and in renal epithelia (16) and otherwise identical to what has been termed system ASC in other cells and tissues (6, 9, 14, 15). Northern blot analysis with the human ATB\(^0\) cDNA-generated probe corroborated the results obtained in the amino acid inhibition profiles and kinetic studies (Fig. 2). A single mRNA species at ~2.9 kb was detected in all six human hepatoma lines under study and in THLE-5B but not in normal human hepatocytes. The 2.9-kb mRNA size and lack of detectable expression in the liver are consistent with results obtained in the original isolation of this cDNA (30). Also illustrated in Fig. 2 is the independence of ATB\(^0\) expression from the differentiation state of the cell line, as both AFP- or albumin-positive and -negative cells express this transporter gene. Moreover, this transporter gene was expressed independent of IGF-II, an oncofetal hormone hypothesized to play a role in hepatocarcinogenesis (46).

RT-PCR analysis of ATB\(^0\) expression. The RT-PCR analysis shown in Fig. 3 confirms that the 2.8-kb mRNA species obtained in Northern blot analysis was indeed ATB\(^0\). RNA isolated from the six hepatoma cell lines, THLE-5B, and JAR-1 [the choriocarcinoma cell line from which the ATB\(^0\) cDNA was isolated (30)] yielded the expected 507-bp RT-PCR product, which, in turn, produced the anticipated 277-, 191-, and 39-bp products on digestion with Sal I and 330-, 114-, and 63-bp products on digestion with Rsa I (not shown). When combined with the Northern blot analyses, the data indicate that the ATB\(^0\) gene product probably underlies the relatively high system ASC glutamine uptake rates in human hepatoma and immortalized liver epithelial cells compared with adult and fetal hepatocytes. Accordingly, we will hereafter refer to the cognate activity as "ASC/B\(^0\)"-mediated transport.
Expression of ATB0 in clinical liver and liver tumor biopsies. All studies to this point had been carried out in human liver cancer cell lines, but we wanted to assess whether the ATB0 gene was expressed in human liver tumors in vivo. To this end, Northern blot analysis was performed on total RNA from human liver and liver tumor biopsies. In a commercially available human liver tumor blot (Northern Territory, Invitrogen), ATB0 mRNA was detectable in human hepatocellular carcinoma (HCC) samples but not normal liver from the same patient in two of three samples (Fig. 4A). To determine the relative level of ATB0 mRNA in HCC, a direct comparison was made with SK-Hep in a Northern analysis. The expression levels of ATB0 mRNA in HCC were not as high as in SK-Hep cells (Fig. 4B), but in a hepatoblastoma biopsy, this transporter gene was expressed at levels severalfold higher than in the hepatoblastoma cell line HepG2 (Fig. 4C). Expression of this glutamine transporter in the nontumorigenic SV40-immortalized THLE-5B liver epithelial cell line (Figs. 1 and 2) raised the possibility that human liver cell growth alone may be sufficient for its transcriptional activation. However, ATB0 mRNA was undetectable in human fetal liver (Fig. 4D), corroborating previous results that showed fetal hepatocytes primarily use system N for glutamine uptake (6). Collectively, these results suggest that ATB0 mRNA is expressed at variable levels in adult and pediatric primary liver cancers, but the growth-related signals that elicit its expression require further investigation.

Expression of ATB0 in fibroblasts and cirrhotic liver. Previously, it was believed that ATB0 expression was restricted to epithelial cells (16, 30). When glutamine uptake was characterized in human fetal liver-derived fibroblasts (HFLF), a profile nearly identical to that in the human hepatoma cells was observed (Fig. 5A), including 94% Na+ dependence, with marked inhibition by alanine, serine, and cysteine but not by MeAIB. Despite the well-established expression of system A in human fibroblasts (22), the contribution of this transporter(s) to glutamine uptake does not become appreciable until extensive depletion of intracellular amino acids is performed (15); conditions not used in this study. Nonlinear regression analysis of the Na+–dependent transport data yielded high \( K_m = 163 \mu M \), \( V_{max} = 5.3 \text{nmol–mg protein}^{-1}\cdot \text{s}^{-1} \) and low-affinity \( K_m = 1.2 \mu M \), \( V_{max} = 3.1 \text{nmol–mg protein}^{-1}\cdot \text{s}^{-1} \) components (Fig. 5A, inset). The high-affinity component, when combined with the amino acid inhibition data, is consistent with what has been described as system ASC, whereas the low-affinity component is assumed to be system A based on past studies with human fibroblasts (15, 17).

A single 2.9-kb ATB0 mRNA species was detectable in the fibroblasts with Northern blot analysis (Fig. 5B). RT-PCR analysis confirmed the identity of the ATB0 mRNA in HFLF and cirrhotic liver, as evidenced by the 507-bp product and subsequent Sal I digestion species of 277, 191, and 39 bp (Fig. 5C). When examined in biopsies from a patient with both liver cirrhosis and HCC, ATB0 mRNA was more abundant in the fibrous noncancerous tissue than in the tumor (Fig. 5D), further suggesting use of this transporter by mesenchymal cells. Glutamine transporter system N (SN1) mRNA was equally expressed in both samples, likely attributable to the presence of normal parenchymal epithelia in each (histology...
not shown), although in situ hybridizations will be required to definitively assign specific transporter expression to individual cell types in biopsies. These data suggest that the ATB0 gene product probably underlies “system ASC”-mediated glutamine uptake in fibroblasts and that this transporter gene is expressed in mesenchymal as well as epithelial cells.

**Role of ATB0 in hepatoma cell proliferation.** The essential role of glutamine in supporting the growth of cells in culture has been well accepted since the pioneering work of Eagle (18). Previously, competitive inhibition of ASC/B0-mediated glutamine uptake in SK-Hep cells with excess alanine, serine, and threonine in the culture media was shown to arrest growth (7). To assess the role of the ATB0 transporter in mediating glutamine-dependent growth in the additional five hepatoma cell lines, each was maintained in media containing specific concentrations of L-glutamine in the absence or presence of excess alanine, threonine, and serine, (6.7 mM each, 20 mM collectively). While these amino acids are also system A substrates, there is no evidence for any MeAIB-inhibitable glutamine transport in these cell lines under the amino acid-rich conditions used in this study (Fig. 1). The results shown in Fig. 6 revealed that two of the hepatoma cell lines (PLC/PRF/5 and FOCUS) were likewise sensitive to substrate-dependent ASC/B0 inhibition in the presence of physiological levels (0.5 mM) of L-glutamine, whereas the Hep3B, HepG2, and Huh-7 were not. When the ambient L-glutamine concentration was raised to normal tissue culture levels of 2 mM, the negative effects of the three ASC/B0 substrates on growth were alleviated.

The individual responses to competitive transporter inhibition were unrelated to the relative glutamine requirements of each cell line for growth. The approx-
iminate concentration of glutamine that supported half-maximal growth (ED50; in mM) for each cell line was as follows: sensitive lines: SK-Hep, 0.3 mM; FOCUS, 0.2 mM; and PLC/PRF/5, 0.3 mM; and refractory lines: Hep3B, 0.1 mM; HepG2, 0.4 mM; and Huh-7, 0.1 mM. Furthermore, the growth responses to competitive ASC/B0 substrate inhibition were independent of growth rates. The doubling times for the sensitive lines were as follows: PLC/PRF/5, 53 h; FOCUS, 23 h; SK-Hep, 18 h. In the refractory lines doubling times were as follows: Hep3B, 32 h; HepG2, 22 h; Huh-7, 20 h. It is interesting to note that the Hep3B, HepG2, and Huh-7 grow in the absence of glutamine, albeit at markedly attenuated rates, over the first week in culture (Fig. 6). When examined under light microscopy, however, the cells were clearly stressed (not shown). Nonetheless, the results indicate that three of six of the human hepatoma cell lines under study appear to largely rely on the ASC/ATB0 transporter to meet the growth-related demands for glutamine.

Northern analysis of GS and other glutamine transporters. A basis for the differential reliance on the ASC/ATB0 transporter for growth in the hepatoma cell lines was provided by Northern blot analysis of other glutamine transporters and GS. The expression of these potential compensatory genes was investigated because they might circumvent the effects of blunted glutamine uptake via ATB0 employed in the growth study. Despite relatively equal ATB0 mRNA expression, the hepatoma cells under study displayed a wide range of GS, system N (SN1 and SN2) and system A (ATA2 and ATA3) isoform mRNA levels (Fig. 7).

GS mRNA was more abundant in the resistant hepatoma cell lines [Huh-7 (1.0) > Hep3B (0.62) > HepG2 (0.39)] vs. the sensitive cell lines [PLC/PRF/5 (0.22) > SK-Hep (0.09) > FOCUS (0.02)], whereas SN1 mRNA was only evident in the resistant cell lines (Huh-7 = Hep3B > HepG2). A 2.5-kb mRNA for system N transporter SN2 was markedly expressed only in HepG2, whereas a less-abundant 3.2-kb SN2 mRNA species was expressed only in Huh-7 and Hep3B. On the basis of these results, it is possible that the Huh-7, HepG2, and Hep3B have a greater capacity to synthesize glutamine endogenously (from glutamate, ATP, and ammonia) and are therefore less dependent on transport for its supply. These three cell lines also apparently have the option of supplying cellular glutamine demands via system N-mediated transport.

The system A transporter ATA2 mRNA (6.1 kb) was expressed in all cell lines under study with Hep3B showing the highest expression levels (Hep3B > HepG2 > SK-Hep > FOCUS > Huh-7 > PLC/PRF/5). In contrast only, the mRNA for the ATA3 isoform was detectable only in the Hep3B (5.5 kb) and Huh-7 (5.5 and 4.9 kb) cell lines, although this cDNA was originally isolated from a HepG2 cDNA library (23).
nonhepatoma THLE-5B cell line expressed only ATA2 in addition to ATB0, and possibly SN1 at very low levels, whereas normal human liver and/or hepatocytes expressed SN1, SN2, ATA2, and ATA3 isoforms at appreciable levels but not ATB0 (Fig. 7). Thus cell lines sensitive to ATB0-inhibited growth arrest (Fig. 6) express only ATA2 in addition to ATB0 for Na+-dependent glutamine uptake. Under the conditions used in this study, ATA2 would be transinhibited, rendering it of little compensatory use in the uptake of glutamine from the media when ATB0-mediated glutamine transport is competitively inhibited with excess substrates.

In summary, the Northern analysis data indicate that the cell lines resistant to ATB0-targeted growth arrest and, to some extent, outright glutamine deprivation (HepG2, Hep3B, and Huh-7 (Fig. 6)) have more compensatory mechanisms at their disposal for glutamine supply than do the poorly differentiated hepatomas (SK-Hep, FOCUS, and PLC/PRF/5).

**DISCUSSION**

Given the potentially important role of glutamine in oncogenesis (39), the role of the liver and plasma membrane transport in glutamine homeostasis (27), and the observed depression of plasma glutamine levels in patients with liver cancer (29), the studies presented here were undertaken to determine whether a switch from...
system N to system ASC for glutamine uptake (6, 9) is a global or consistent feature of transformed human liver cells and to identify the gene responsible for this accelerated activity. The results demonstrate that relatively high rates of system ASC-mediated glutamine transport are a consistent feature of all six human hepatoma lines studied to date and that the product of the ATB0 gene, whose functional characteristics match those described for ASC-mediated glutamine uptake (6, 12, 30), is probably responsible for this activity. This conclusion is based on marked inhibition of glutamine uptake by alanine, cysteine, and serine relative to the other amino acids tested, including the system A-specific substrate MeAIB (Fig. 1) and the confirmed expression of this transporter via Northern blot and RT-PCR (Figs. 2 and 3). Although other transporters are expressed in some of the cell lines (Fig. 7), on the basis of the kinetic analyses, the high-affinity component (ATB0) mediates >80% of glutamine uptake at initial-rate concentrations (50 μM), and 60–70% of uptake at physiological concentrations (600 μM) in the hepatomas and THLE-5B. An additional finding from these studies is that ATB0 is largely responsible for glutamine uptake in fibroblasts (Fig. 5). This is significant in that it was previously thought that ATB0 expression was restricted to epithelial cells (16, 31). Thus the “system ASC” activity described in human fibroblasts for the past 20 years (12, 15, 22) is probably ATB0. The deduced amino acid sequence of human ATB0 gene is highly homologous (79–85% amino acid identity) to that encoded by rabbit ATB0 and mouse and rat ASCT2 (10, 31, 51). On the basis of cross-species cDNA library screenings and nearly identical functional characteristics, it has been concluded that ASCT2 and ATB0 represent interspecies orthologous isoforms of the same transporter (5, 11). Thus investigators might consider referring to this transporter as ASCT2 in all mammalian species. ATB0/ASCT2 is a member of the excitatory amino acid transporter family, which includes the glutamate transporters and another system ASC isoform (ASCT1) that does not transport the amides asparagine or glutamine (3, 48).

Although it is clear that ATB0/ASCT2 mediates the majority of glutamine uptake in proliferating human liver-derived cells in vitro, signals for its transcriptional activation are poorly understood. Results in the immortalized nontumorigenic human liver epithelial cell line THLE-5B (45) suggest that its expression is not limited to tumor-derived liver cells (Figs. 1–3). In the original cloning and characterization paper, ATB0 was expressed in a number of normal human tissues, but its mRNA was not detectable in liver by Northern blot analysis (30). The results in the present study corroborate those findings, because ATB0 mRNA is undetectable by Northern blotting in isolated human hepatocytes and adult and fetal human liver (Figs. 2 and 4), cells and tissues that primarily use system N rather than system ASC/B0 for glutamine transport (6, 35). The lack of detectable mRNA and activity in fetal human liver (Fig. 4D and Ref. 6) also indicates that liver cell growth per se is not alone sufficient to elicit ATB0 expression. Analysis of its expression in regenerating human liver would provide a better assessment of its activation as a result of hepatocyte cell cycle activation, but unfortunately, no good source of regenerating human liver was available, with the possible exception of the cirrhotic liver sample in Fig. 5. In fact, no cellular system has yet been identified whereby ATB0 expression can be elicited from a previously dormant background, but previous work from our laboratory has shown a relationship between relative rates of ASC/B0-mediated glutamine transport activity and
growth or growth-related processes. For example, chemically arrested hepatoma cells exhibit diminished glutamine uptake rates (9), whereas ASC/B0-mediated glutamine transport regulates DNA and protein synthetic rates in tumor cells (52). ASC/B0 activity has also been shown to fluctuate in a cell cycle-dependent manner in SK-Hep cells, ~40% higher in G1/S relative to G2/M (43). Recent work also showed that ASC/B0-mediated glutamine uptake increases threefold when SK-Hep cells are grown as large multicellular spheroids (44) and that chronic downregulation of ASC/B0 activity with phorbol esters arrests the growth of this cell line (7). Despite these links between growth and glutamine transport activity, identification and study of the promoter region of the ATB0 gene will be required to address the mechanism of its activation in hepatoma and immortalized liver cells vs. lack of expression in fetal liver.

To test the clinical relevance of our results obtained in human liver tumor cell lines, Northern blot analysis showed that hepatocellular carcinomas expressed ATB0 mRNA, albeit at levels less than the HCC cell line SK-Hep (Fig. 4B). This could be the result of “dilution” of hepatoma RNA by normal hepatocyte RNA in the biopsy. It should be noted that the HCC samples shown in Figs. 4B and 5D are from two separate patients and contain approximately equal ATB0 mRNA levels. In contrast to HCC, the hepatoblastoma biopsy contained dramatically more ATB0 mRNA than the hepatoblastoma cell line HepG2 (Fig. 4C). However, it is difficult to assess whether relative mRNA levels directly correlate to ASC-mediated glutamine uptake rates. Clearly, a direct correlation between ATB0 mRNA levels and transport velocities was not apparent in the cell lines under study, as evidenced by the marked transport velocities in the THLE-5B cell line relative to the others (Figs. 1 and 2). Such disparate activities in the face of comparable ATB0 mRNA levels further implicate significant translational and established posttranslational mechanisms [membrane potentials, intracellular amino acid levels (11, 31, 50)] in determining the measured rates of glutamine uptake via this carrier. Antibodies against the ATB0 protein, currently in development in our laboratory, will help to resolve this issue in future studies and will aid in the identification of ATB0-positive cells within tumor biopsies. Nonetheless, our results indicate that the ATB0 gene is expressed in liver tumors as well as liver tumor-derived cell lines.

With respect to the main finding in these studies of the ubiquity of ATB0 expression in hepatoma cells, why might a glutamine transporter switch from system N to system ASC/B0 be advantageous? The data presented in Fig. 6 suggest that some of the hepatoma cells rely more heavily on this transporter to drive glutamine-dependent growth than others with a greater capacity to produce glutamine endogenously or the ability to take it up by alternative routes such as SN1 or SN2 (Fig. 7). This finding may be significant given the low GS activity observed in human liver tumors (37, 38). One practical consequence of ATB0 gene expression is that it allows cells to take up glutamine more efficiently than system N or A at relatively low ambient concentrations because of its higher affinity for this substrate (Fig. 1). A lower $K_m$ may prove effective in a poorly vascularized environment such as a tumor mass or connective tissue where glutamine concentrations may be diminished. Ultimately, different transport mechanisms between systems N and ASC/B0 may underlie the heightened activity and preferential expression of the latter in human hepatoma cells. System ASC activity is known to be enhanced by “transstimulation,” a mechanism whereby increased levels of intracellular amino acid substrates accelerate the observed inward transport velocity of others (12, 22). ATB0 has recently been characterized by electrophysiological techniques and found to mediate Na+-dependent amino acid exchange rather than net uptake (50). Na+-dependent amino acid exchange allows cells to equilibrate pools of most zwitterionic amino acids via a “trade” of relatively abundant intracellular substrates for more coveted extracellular ones. Through this mechanism, ATB0 would indeed support growth and mediate the net uptake of glutamine, because faster growing hepatomas tend to have proportionally lower intracellular glutamine levels, at least in rodents (47), although no data are currently available on intracellular glutamine concentrations in the human hepatoma cell lines. Such an equilibrating mechanism may better lend itself to hepatoma growth than a narrow specificity carrier such as system N, whose properties are geared more toward systemic glutamine economy (5, 19). However, the mechanistic and cell-specific basis for ATB0 gene activation must be elucidated before the teleological aspects of this problem can be soundly addressed.

To date, three isoforms of system A and two isoforms of system N have been identified, and all belong to the same transporter family (5). We examined the expression of both system N isoforms and two of the three system A isoforms (ATA2 and ATA3) that are most liver specific (23, 25). The ATA1 isoform appears to exhibit more brain-specific expression, although its mRNA has been detected in HepG2 cells (24), so its potential contribution to glutamine uptake in the cell lines under study here cannot be discounted. When the kinetic data are compared with the results with Northern blots (Fig. 7), the low-affinity component is probably ATA2 in the cell lines sensitive to ATB0-competitive growth inhibition [SK-Hep, PLC/PRF/5, and FOCUS (Fig. 6)], because no SN1, SN2, or ATA3 mRNA was detectable in these cells. Under the conditions used in the growth inhibition studies, ATA2 would be both transinhibited and competitively inhibited from taking up glutamine due to excess alanine, serine, and threonine in the media and would therefore serve little compensatory role in relieving the attenuated glutamine supply. In contrast, the cell lines resistant to ATB0 competitive growth inhibition [HepG2, Hep3B, and Huh-7 (Fig. 6)] express both system N isoforms as well as ATA2 and ATA3 in the Hep3B and Huh-7. The low-affinity component for Na+-dependent
 glutamine uptake is probably a collective contribution of the system A and N isoforms, because all exhibit deduced $K_m$ values of 1–3 mM for glutamine (19, 23, 25, 42). Alanine has recently been shown to be a marginal SN1 substrate (19), whereas serine has been shown to be a strong SN2 substrate (42), but neither amino acid has the capacity to completely block system N-mediated glutamine uptake. Moreover, the resistant cell lines grow at reduced rates for a brief period in the total absence of glutamine (Fig. 6); two of these three cell lines (Hep3B and Huh-7) also exhibited the lowest total absence of glutamine (Fig. 6); two of these three cell lines grow at reduced rates for a brief period in the

25, 42). Alanine has recently been shown to be a mar-
to the three lines sensitive to ATB0 inhibition (Figs. 6

to 50-fold more abundant in these cell lines relative
glutamine could be provided by GS, whose mRNA is 2-
resulting in a marginal glutamine metabolic economy in
these cells. Under glutamine deprivation, intracellular

glutamine could be provided by GS, whose mRNA is 2-
to 50-fold more abundant in these cell lines relative
to the three lines sensitive to ATB0 inhibition (Figs. 6

and 7).

In summary, this study demonstrates that the ATB0
gene product largely mediates the accelerated rates of
hepatoma cell line glutamine uptake reported here and
previously (6, 9) as well as in human fibroblasts. The
data also suggest that ASCT2/ATB0 may govern the
growth of poorly differentiated human liver tumor-
derived cells lacking system N expression and a limited
capacity to produce glutamine endogenously, similar to
HCC in vivo (37, 38). On the basis of the differential
expression of ATB0 mRNA in the hepatocellular carci-
noma samples and hepatoblastoma, it is possible that
this transporter plays a role in the development and
growth of certain liver tumors. Targeted molecular
inhibition of ATB0 expression, work that is currently
in progress in our laboratory, will provide further in-
sights into the role of this glutamine transporter in
hepatoma cell growth and survival.

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