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Overexpression of LAT1/CD98 Light Chain Is Sufficient to Increase System L-Amino Acid Transport Activity in Mouse Hepatocytes but Not Fibroblasts*

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William A. Campbell and Nancy L. Thompson‡

From the Division of Medical Oncology, Brown University School of Medicine and Graduate Program in Pathobiology, Rhode Island Hospital, Providence, Rhode Island 02903

L-amino acid transporter-1 (LAT1) is a highly conserved gene identified as a light chain of the CD98 amino acid transporter and cellular activation marker. In our previous studies we found increased expression of LAT1 in primary human cancers. We have demonstrated also that LAT1 response to arginine availability is lost in transformed and tumorigenic cells such that expression is constitutively high. System L-amino acid transport activity correlates with changes in LAT1. To assess the functional relevance of increased LAT1 expression and the requirement for 4F2 heavy chain, we overexpressed these CD98 subunits together and separately in nontransformed hepatocytes and fibroblasts. Antigen tags in the expression constructs confirmed that expressed proteins were localized to both cytoplasmic and plasma membrane locations within the cells. Overexpression of LAT1 alone in mouse hepatocytes, but not fibroblasts, was sufficient to increase system L transport, and these cells displayed a growth advantage in conditions of limited arginine. Our results suggest that loss of regulation leading to constitutive expression of LAT1 can contribute to oncogenesis. We hypothesize that the altered LAT1 expression observed in hepatocarcinogenesis gives cells a growth or survival advantage through increased transport activity in a tumor microenvironment of limited amino acid availability.

LAT1/TA1 and its homologs have been identified by multiple investigators as one of several alternate light chains of the CD98 amino acid transporter and cellular activation marker (1–6). TA1 was cloned in our laboratory on the basis of its differential expression between rat hepatoma cells and normal adult rat liver and is identical to the C terminus (amino acids 272–512) of LAT1 (7). The CD98 complex consists of an 80-kDa heavy chain (4F2hc) and a 40–45-kDa light chain (8). CD98 has been implicated in a variety of functions including amino acid transport, cell survival, integrin activation, and cell fusion (9–12). Various light chains have the potential to form a complex with heavy chain depending on the cell type and intracellular localization. Additional light chains have been identified and designated y^+LAT-1 (13–15), y^+LAT-2 (13, 16), LAT-2 (17, 18), xCT (19), 4F2-lc6 (20), and ASC-1 (21). The CD98 complex can mediate various amino acid transport systems in *Xenopus* oocytes depending on which CD98 light chain (CD98lc) is associated with the CD98 heavy chain (CD98hc) (3–6, 13). Coinjection of cRNA for TA1/LAT-1 and 4F2hc has been shown to mediate system L transport of large neutral amino acids with branched or aromatic side chains in *Xenopus* oocytes (3, 5).

We have found increased levels of LAT1 mRNA in both rat and human carcinomas and in the carbon tetrachloride model of liver injury/regeneration (7, 22–24). Adaptive regulation of message for LAT1 but not 4F2hc was observed in response to arginine levels in normal hepatic cells (25). Loss of LAT1 regulation with corresponding increased system L transport was associated with malignant progression, suggesting that upregulation of LAT1 or functional CD98 complex contributes to the neoplastic phenotype. Hara *et al.* (26) have shown that NIH3T3 cells transfected to overexpress 4F2hc demonstrated classic properties of malignant cells including higher saturation density, growth in soft agar, and tumor formation in athymic mice. Researchers from the same laboratory demonstrated more recently that transformation of BALB3T3 cells by overexpression of 4F2hc requires its association with an unidentified light chain (27).

We are interested in the regulation and role of LAT1 and CD98-related molecules in transformation and carcinogenesis. To examine the relationship of cell type to overexpression of these molecules and associated transport properties, we transiently overexpressed LAT1 and 4F2hc alone or together in nontransformed mouse hepatocyte and fibroblast cell lines. We found that overexpression of LAT1 alone was sufficient to increase system L transport activity significantly in the epithelial cells but not in the fibroblasts. In contrast, increases in transport activity in fibroblasts required coexpression of both LAT1 and 4F2hc. Hepatic cells overexpressing LAT1 displayed a growth advantage relative to control cells under conditions of limited arginine. We postulate that differences in LAT1 expression and response to amino acid availability influence cell growth and survival properties and that the LAT1-CD98 pathway may represent a unique therapeutic target for cancer intervention.

EXPERIMENTAL PROCEDURES

Cell Culture—AML12 cells, a normal mouse hepatocyte line, were provided kindly by Dr. Nelson Fausto (Department of Pathology, University of Washington) and maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Life Technologies, Inc.) containing 10% fetal bovine serum and supplemented with insulin, transferrin, and selenium (ITS^+) at 1 ml/liter medium (Becton Dickinson) and 0.1 μM dexamethasone. NIH3T3 cells were cultured routinely in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. A custom

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‡ To whom correspondence should be addressed: Medical Oncology, Rhode Island Hospital, 593 Eddy St., Providence, RI 02903. Tel.: 401-444-8860; Fax: 401-444-8141; E-mail: Nancy_Thompson@brown.edu.

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formulation of Chee's essential medium (CEM)[1] that was demonstrated to maintain long term differentiated cell function *in vitro* was provided kindly by Dr. Hugo Jauregui (Department of Pathology, Rhode Island Hospital) and prepared as described previously (28). Arginine was not present in this custom formulation and was added from stock solutions. For experiments in which gene expression was assayed as a function of arginine availability, cells were seeded into T-75 flasks with CEM containing 5% fetal bovine serum with and without arginine (25). Previous experiments have shown no difference in results with dialyzed or nondialyzed serum (25). Medium was changed every day. Viability was at least 90% in all cases.

Functional Expression of cDNAs in AML12 and NIH3T3 Cells—Rat LAT1 cDNA was subcloned into the pFLAG-5a expression vector (Sigma) and sequenced to confirm the correct sequence and the addition of the FLAG epitope tag. pL(CD98-HA)IZ expression construct and monoclonal antibody HA.11 (Babco) were provided kindly by Dr. Tatiana Kolesnikova and Dr. Martin Hemler (Dana Farber Cancer Institute, Harvard Medical School). pL(CD98-HA)IZ expresses human 4F2hc/CD98 heavy chain with a hemagglutinin epitope tag. pEGFP-F (CLONTECH) encodes farnesylated enhanced green fluorescent protein.

For transport assays, AML12 cells and NIH3T3 cells were seeded, respectively, at 2×10^5 and 7.5×10^4 cells/well of a 24-well plate 24 h prior to transfection. For immunofluorescence, AML12 and NIH3T3 cells were seeded, respectively, at 4×10^5 and 1.5×10^5 cells/chamber of a slide chamber 24 h prior to transfection. Transfection was carried out with LipofectAMINE Plus reagent (Life Technologies, Inc.). For a 24-well plate, 0.6 μg of DNA, 3 μl of Plus reagent, and 1.5 μl of LipofectAMINE were used per well. For a chamber slide, 1.2 μg of DNA, 6 μl of Plus reagent, and 3 μl of LipofectAMINE were used per chamber. The functional expression of each cDNA was analyzed 48 h post-transfection by measuring radiolabeled amino acid uptake and by immunofluorescence in AML12 and NIH3T3 cells. Cotransfection with pEGFP-F was used for every experiment to monitor transfection efficiency by counting fluorescent cells expressing GFP. Transfection efficiency routinely approached 40 and 50% for NIH3T3 and AML12, respectively.

Immunofluorescence—Expression of exogenous LAT1 and 4F2 was monitored by indirect immunofluorescence microscopy using antibodies directed to the epitope tag present on each protein. M2 antibody (Sigma) was used to detect FLAG-tagged LAT1, whereas HA.11 antibody (Babco) was used to detect hemagglutinin-tagged 4F2hc. Briefly, AML12 and NIH3T3 cells were transfected with pFLAG-LAT1 or pL(CD98-HA)IZ, and at 48 h after transfection, slides were fixed in cold acetone. Slides were blocked with 1% normal goat serum in PBS and then incubated with either M2 (mouse anti-FLAG) antibody at 10 μg/ml or HA.11 (mouse anti-hemagglutinin) antibody at 1:100 dilution in 1% normal goat serum in PBS. Slides were washed with PBS, blocked again, and incubated with goat anti-mouse secondary antibody (Sigma) at 1:100 dilution in 1% normal goat serum in PBS. After a subsequent wash and coverslipping, slides were examined on a Nikon Microphot-EPI-FL fluorescence microscope.

Expression of endogenous LAT1 was detected using affinity-purified anti-peptide antibody to the NH_2 terminus of LAT1 at 12 μg/ml. MacVector sequence analysis of the rat LAT1 coding region was used to select a region with little or no homology to other CD98 light chains and with high conservation to human LAT-1 and high hydrophilicity. Peptide 20–30, EERQAREKML, near the NH_2 terminus scored highest for these qualities and was synthesized commercially with an NH_2-terminal cysteine for conjugation to keyhole lympet hemocyanin and used to immunize two rabbits following a conventional immunization schedule (Sigma-Genosys). The specificity of this antibody was demonstrated by absorption with the immunizing peptide (data not shown). Expression of endogenous 4F2hc was detected using an anti-4F2hc rabbit anti-peptide antibody (1:100) kindly provided by Dr. Suresh S. Tate (Cornell University Medical College, NY). This polyclonal antiserum was raised against a 92-amino acid peptide fragment (Lys-114–Tyr-205) of the rat glioma cell 4F2hc (29). Normal rabbit serum at the same concentration or dilution was used as a negative control (data not shown). Normal liver and GP7TB tumor cells were used as negative and positive controls, respectively, for LAT1 antibody reactivity (data not shown).

RNA Preparation and Northern Blot Analysis—Total RNA was isolated using the guanidinium isothiocyanate/cesium chloride method (30) for rat placenta and a modification of that method for cultured cell lines (Totally RNA Kit™, Ambion, Austin, TX). RNA was isolated from cultured cells after subculture in CEM for the relevant time points. Aliquots (12 μg) of total RNA were size-fractionated on 1% agarose/formaldehyde gels as described previously (22). After electrophoresis, gels were equilibrated in 1 M ammonium acetate, and RNA was transferred to Nytran nylon membranes (Schleicher and Schüll). Blots were baked for 2 h at 80 °C and hybridized at 65 °C according to Church and Gilbert (31). Rat *TA1* p900, a fragment corresponding to nucleotides 816–1536 of the full-length rat LAT-1 inserted into Bluescript SK vector, was labeled with [^{32}P]dCTP (3000 Ci/mmol, PerkinElmer Life Sciences) by random primed labeling (Roche Molecular Biochemicals) for use as a probe. Blots wrapped in plastic wrap were exposed to x-ray film (Eastman Kodak Co.) at −70 °C in the presence of intensifying screens. Blots were stripped and rehybridized to an 1800-base pair *Eco*RI fragment of human *4F2hc* (provided by Dr. Martin Hemler) (32). Densitometry using the Quantity One™ IBM software package was used to quantify differences in RNA levels with normalization to 18 S ribosomal RNA.

Amino Acid Transport Assays—The transport of radiolabeled amino acids by cell monolayers was performed using a modification of the cluster-tray method developed by Gazzola *et al.* (33) and described by Kilberg (34). All ^{3}H-labeled amino acids were purchased from PerkinElmer Life Sciences and unlabeled amino acids were purchased from Sigma. Cells were near confluent for the transport assay. Transport assays were performed as described previously (25). Briefly, before the transport assays, cells were rinsed with warm Na^+-free KRP buffer, in which the sodium-containing salts were iso-osmotically replaced with choline, to remove extracellular Na^+ and amino acids. Cells were incubated in warm Na^+-free KRP buffer for 10 min to deplete intracellular amino acids. The uptake of radiolabeled amino acids (5 μCi of [^{3}H]amino acid/ml) at 50 μmol/liter in either 200 μl of Na^+-free KRP buffer or sodium KRP was measured for 30 s at 37 °C. Preliminary experiments indicated that uptake of each ^{3}H-labeled amino acid was linearly dependent on incubation time up to at least 3 min; therefore, uptake was measured for 30 s (data not shown). In inhibition experiments, excess cold 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) was added to the uptake buffer at 10 mM final concentration. Uptake was terminated by washing the cells rapidly four times with 1 ml/well of ice-cold Na^+-free KRP buffer. After the trays were allowed to dry, the cells were incubated for 1 h with 0.2 ml/well of 0.2% (w/v) SDS plus 0.2 N NaOH to release intracellular radioactivity. A 0.1-ml aliquot

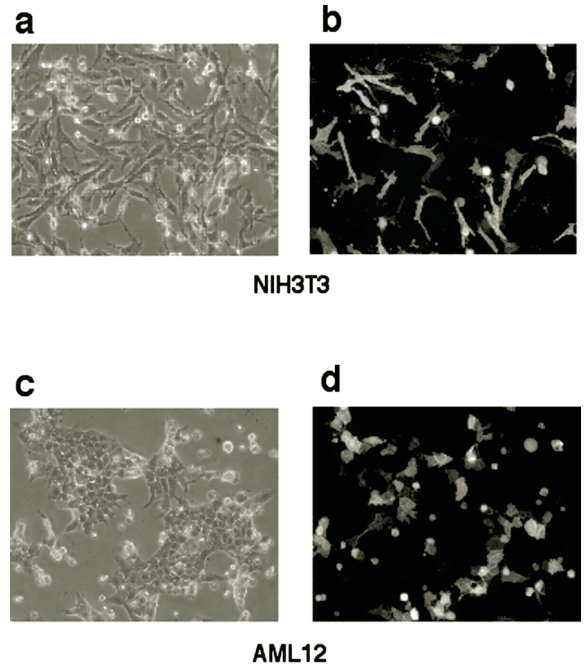

FIG. 1. **Efficiency of transfection in mouse cells.** NIH3T3 and AML12 cells cotransfected with pEGFP-F and pFLAG-5a (empty FLAG vector). NIH3T3 cells (*a* and *b*) and AML12 cells (*c* and *d*) were examined 48 h post-transfection under phase and fluorescence microscopy, respectively. Transfection efficiency was monitored in all experiments by cotransfection with pEGFP-F.

[1] The abbreviations used are: CEM, Chee's essential medium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; KRP, Krebs Ringer phosphate; BCH, 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid; HA, hemagglutinin.

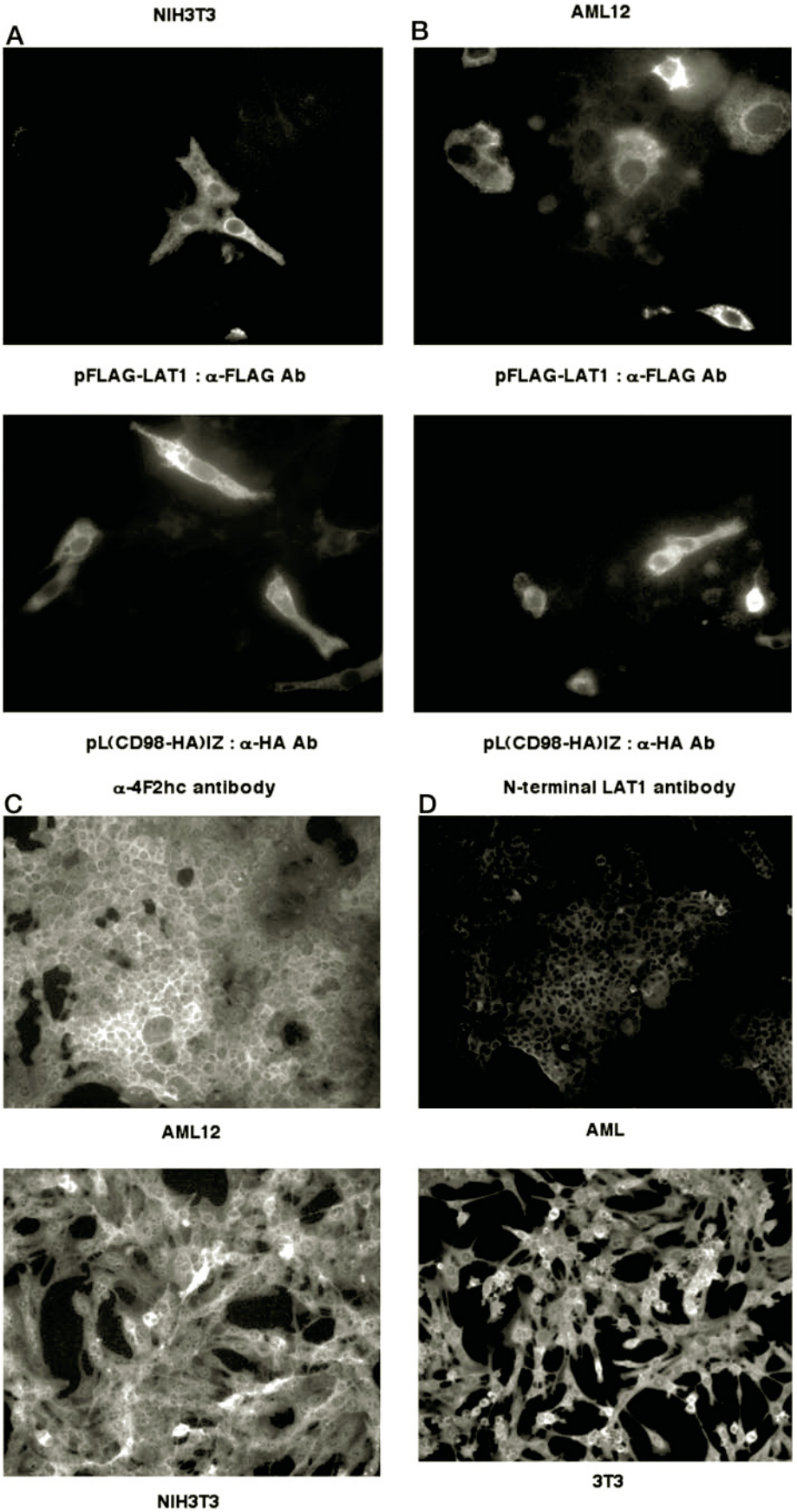

FIG. 2. **Immunofluorescence detection and localization of exogenous and endogenous LAT1 and 4F2hc protein in mouse cells.** *A* and *B*, immunofluorescence using antibody to the epitope tag present in pFLAG-LAT1 or pL(CD98-HA)IZ was performed 48 h after transfection of NIH3T3 (*A*) or AML12 cells (*B*) with each construct. The staining pattern indicated significant colocalization of LAT1 and 4F2hc protein with intense cell surface and cytoplasmic staining observed in both cell types. *C*, endogenous 4F2hc protein was detected in AML12 and NIH3T3 cells using anti-peptide antibody to 4F2hc. *D*, endogenous LAT1 protein was detected in AML12 and NIH3T3 cells using anti-peptide antibody to LAT1. Intense staining for endogenous 4F2hc was observed at the cell surface and in the cytoplasm in both cell types. Intense cell surface and cytoplasmic staining for endogenous LAT1 protein was observed in the NIH3T3 cells, but in contrast, very weak staining for LAT1 protein was observed in AML12 cells. The localization of endogenous and exogenous LAT1 and 4F2hc protein was similar in both cell types.

from each well was neutralized with 0.1 ml of 0.2 N HCl and quantified in a Beckman LS 6000SC liquid scintillation counter. The remaining 0.1 ml was analyzed for protein content using the BCA protein assay reagent (Pierce). Transport velocities were calculated from radioactive counts, specific activities of uptake mixes, and protein absorbance values and were expressed as picomoles amino acid transported per mg of protein per min (averages ± S.E. of ≥ 4 separate determinations). All data were normalized for transfection efficiency. Data comparing two

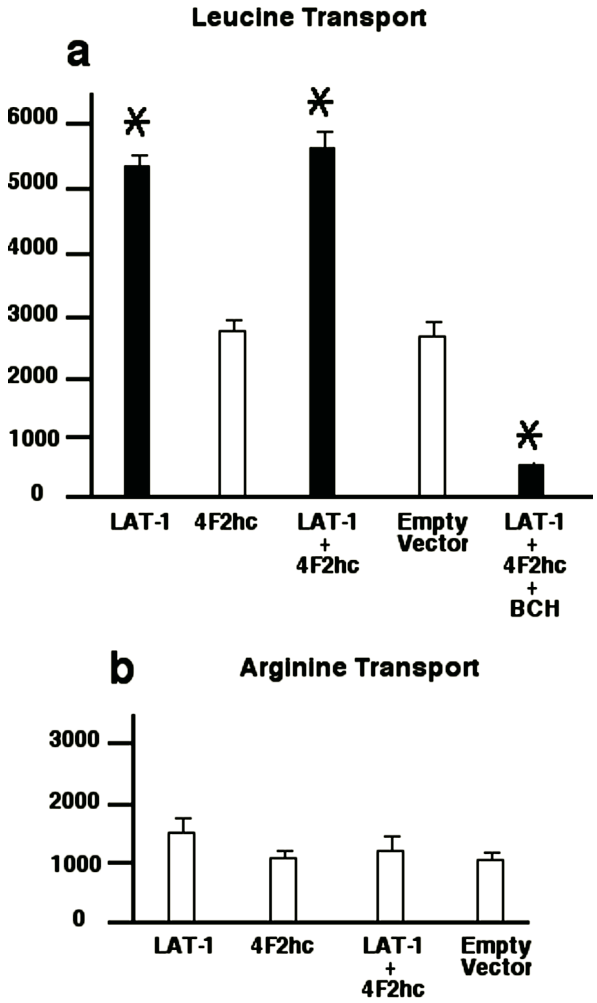

FIG. 3. **Amino acid transport analysis in transfected mouse hepatocytes.** The uptake of 50 μM radiolabeled leucine and arginine was measured in transfected AML12 cells 48 h post-transfection with plasmid DNA. All transport values are expressed as picomoles per mg of protein per min. Transport was found to be sodium-independent (data not shown), so only transport values in the absence of sodium are depicted. Each experiment was repeated at least twice with similar results. Values are means ± S.E. of twelve measurements from three separate experiments. *a*, leucine transport was measured in transfected cells. Excess cold BCH inhibited leucine transport. *b*, arginine transport was measured in transfected cells. Transfection with LAT1 alone significantly increased leucine transport ($p < 0.005$) *versus* transfection with 4F2hc or empty vector. Cotransfection of LAT1 and 4F2hc did not increase transport above that of LAT1 alone. Arginine transport did not vary after transfection with any of the constructs.

experimental results were analyzed statistically by Student's *t* test using the InStat Macintosh statistics program. Two experimental results were considered to be statistically significantly different when $p < 0.05$. Each experiment was repeated with independent transfections at least twice to show qualitatively the same results. Averages of three independent experiments are shown for both AML12 and NIH3T3 cells.

Proliferation Assay and Cell Cycle Analysis—Cells were seeded into 6-well plates, transfected with plasmid DNA, and then cultured in CEM containing 5% fetal bovine serum without arginine. The medium was changed every day. Transfection efficiency was monitored by GFP expression and immunofluorescence. Cells were counted by the trypan dye exclusion test at the beginning of the experiment as well as 24 and 48 h after culture in CEM without arginine. Cell number was expressed as averages ± S.E. of three or more separate determinations. The experiment was repeated with independent transfections to show qualitatively the same results. An aliquot of cells from each condition was saved for cell cycle analysis. These cells were stained with 1× propidium iodide stain (50 μg/ml propidium iodide, 0.1% Nonidet P-40, and 0.1% sodium citrate in H_2O) for ~10 min. Cell cycle analysis was performed using a Becton Dickinson FACSORT flow cytometer, and data were analyzed with Cell Quest software.

RESULTS

Transfection of AML12 and NIH3T3 Cells—AML12 cells, an immortalized, nontransformed, nontumorigenic, well differentiated hepatocyte cell line, was established from the liver of transgenic mice overexpressing transforming growth factor-α (35). This cell line was established because hepatocytes cannot be maintained for long periods of time as replicating, differentiated cells while remaining nontumorigenic. These cells have been used extensively as a model to study tissue-specific gene regulation and hepatocarcinogenesis because they are easily transfectable, do not display anchorage-independent growth or form tumors in nude mice, and maintain expression of differentiated hepatocyte markers after extensive passaging (35–37). NIH3T3 cells have been utilized by other investigators (26) to examine the functional significance of CD98 heavy chain overexpression. AML12 and NIH3T3 cells were cotransfected with pEGFP-F and pFLAG-5a, and transfection efficiency was monitored 48 h post-transfection by fluorescence of GFP (Fig. 1). Transfection efficiencies routinely approached 50 and 40% for AML12 and NIH3T3, respectively. Cotransfection with pEGFP-F was used to monitor transfection efficiency in all amino acid transport experiments.

Immunofluorescence Detection of Exogenous and Endogenous LAT1 and 4F2hc in NIH3T3 and AML Cells—NIH3T3 and AML12 cells were transfected with either pFLAG-LAT1 or pL(CD98-HA)IZ to verify expression of exogenous protein by immunofluorescence in addition to transport activity (Fig. 2, *A* and *B*). Both FLAG-tagged LAT1 and hemagglutinin-tagged 4F2hc were detected in transfected NIH3T3 and AML12 cells. The staining pattern indicated significant colocalization in both cell types. Staining was observed at the cell surface as well as within the cytoplasm. Anti-peptide antibodies were used to detect endogenous 4F2hc and LAT1 staining in both AML12 and NIH3T3 cells (Fig. 2, *C* and *D*). The LAT1 epitope is on the NH_2 terminus of the molecule, which is predicted to be cytoplasmic (38). The antibody to 4F2hc is directed to a predicted ectodomain (38). Intense staining was observed for 4F2hc in AML12 and NIH3T3 cells and for LAT1 in NIH3T3 cells. Staining for LAT1 was very weak in AML12 cells. The staining pattern for endogenous 4F2hc and LAT1 was very similar to staining for exogenous 4F2hc and LAT1 with both cytoplasmic and cell membrane staining. We observed similar immunofluorescent staining for all transfections.

Overexpression of LAT1 Is Sufficient to Increase System L Transport Activity in AML12 Cells—In previous studies using a panel of hepatic cell lines with specific differences in transformation and tumorigenicity, we found that LAT1 but not 4F2hc correlated with transformation, tumorigenicity, and increases in system L transport activity (25). To determine the functional relevance of overexpression of LAT1 and requirement for 4F2hc in nontransformed hepatocytes, we overexpressed LAT1 and/or 4F2hc in AML12 cells and measured transport activity. Transport activity for systems L and y^+ were found to be sodium-independent (data not shown). As shown in Fig. 3*a*, overexpression of LAT1 increased system L-specific transport activity 2-fold (significant at $p < 0.005$) relative to 4F2hc or empty vector transfection. Overexpression of LAT1 and 4F2hc did not increase transport activity above that of LAT1 alone. As expected, addition of excess cold BCH inhibited leucine transport, consistent with system L activity. Arginine transport (system y^+) was not significantly different in any of the transfection conditions (Fig. 3*b*) and served as a control for any nonspecific influence of the transfection conditions.

Overexpression of LAT1 and 4F2hc Is Necessary to Increase

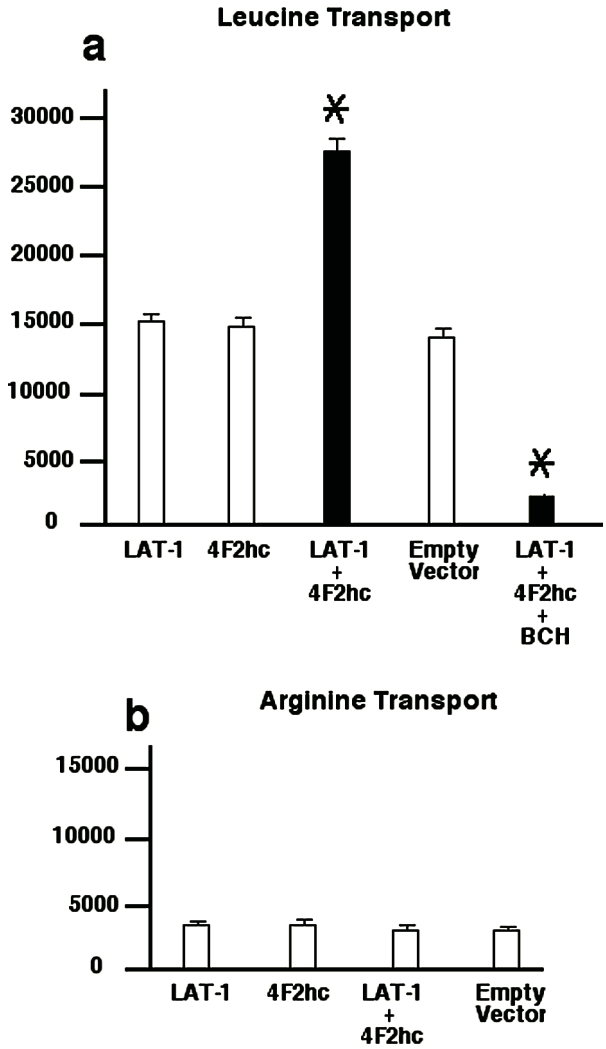

FIG. 4. **Amino acid transport analysis in transfected mouse fibroblasts.** The uptake of 50 μM radiolabeled leucine and arginine was measured in transfected NIH3T3 cells 48 h post-transfection with plasmid DNA. All transport values are expressed as picomoles per mg of protein per min. Transport was found to be sodium-independent (data not shown), therefore only transport values in the absence of sodium are depicted. Each experiment was repeated at least twice with similar results. Values are means ± S.E. of twelve measurements from three separate experiments. *a*, leucine transport was measured in transfected cells. Excess cold BCH inhibited leucine transport. *b*, arginine transport was measured in transfected cells. Cotransfection of LAT1 and 4F2hc significantly increased leucine transport ($p < 0.005$) *versus* single transfection with LAT1, 4F2hc, or empty vector. Arginine transport did not vary after transfection with any of the constructs.

System L Transport Activity in NIH3T3 Cells—Other investigators have demonstrated that overexpression of CD98 heavy chain/4F2hc transforms NIH3T3 cells and that transformation depends on association with an unidentified light chain (26, 27). To determine whether LAT1 transfection can increase transport activity in NIH3T3 cells and to compare activity in hepatocytes *versus* fibroblasts, we overexpressed LAT1 alone or with 4F2hc in NIH3T3 cells. We found that overexpression of both LAT1 and 4F2hc increased system L-specific transport activity approximately 2-fold (significant at $p < 0.005$) relative to LAT1, 4F2hc, or empty vector transfection (Fig. 4*a*). In contrast to the studies in hepatocytes, however, overexpression of LAT1 alone had no significant effect on transport activity. As expected, the addition of excess cold BCH inhibited leucine transport, which is consistent with system L activity. Arginine transport (system y^+) was not significantly different after any of the transfections (Fig. 4*b*). Interestingly, system L amino acid transport activity was 3–6-fold higher in NIH3T3 cells compared with AML12 cells on a transport activity per cell basis (Table I).

TABLE I
Relative System L amino Acid transport activity in transfected cells

Transfected DNA	AML12	NIH3T3
LAT1	2	6
4F2hc	1	6
LAT1 and 4F2hc	2	11
Empty vector	1	6

Endogenous LAT1 and 4F2hc RNA Levels in AML12 and NIH3T3 Cells—In previous studies we have seen adaptive regulation of message for LAT1 but not CD98 heavy chain/4F2 in response to arginine levels in normal but not transformed or tumorigenic hepatic cells (25). We were interested in whether the response to arginine of LAT1 and 4F2hc message in AML12 and NIH3T3 cells resembled the response of normal or transformed cells. We also wanted to determine whether endogenous LAT1 and 4F2hc levels may contribute to the observed differences in transport activity after transfection between AML12 and NIH3T3 cells. Immunofluorescence data (above) indicated intense staining for 4F2hc in both AML12 and NIH3T3 cells and for LAT1 in NIH3T3 cells but relatively weak staining for LAT1 in AML12 cells. AML12 and NIH3T3 cells were cultured for 24 h in CEM with or without arginine. Steady-state LAT1 mRNA levels were very low in AML12 cells cultured in medium with arginine but induced 5-fold in the absence of arginine (Fig. 5). Similar results were obtained after 48 h of culture. LAT1 mRNA levels were constitutively high in NIH3T3 cells regardless of arginine availability. Although basal levels of LAT1 message were much higher in NIH3T3 cells relative to AML12 cells, 4F2hc steady-state message levels were high and nonresponsive to arginine availability in both cell types. Under the conditions of the transport assays, *i.e.* in the presence of arginine, LAT1 message is low in AML12 cells relative to NIH3T3 cells, whereas 4F2hc message levels were equivalent in the two cell types. Thus, message levels correlate well with the observed LAT1 and 4F2hc immunofluorescence.

Proliferation of LAT1-transfected AML12 Cells Cultured in Conditions of Limiting Arginine—LAT1 expression is up-regulated in nontransformed hepatocytes after culture without arginine, and loss of that regulation and constitutive expression of LAT1 is associated with the transformed phenotype (25). We have hypothesized that up-regulation of LAT1 confers a growth or survival advantage in the tumor microenvironment. To determine whether AML12 cells overexpressing LAT1 had a growth advantage in limiting arginine conditions over AML12 cells expressing endogenous LAT1, AML12 cells were transfected with either pFLAG-LAT1 or pEGFP-F (as a control and monitor of transfection efficiency). The medium was changed to CEM without arginine immediately after transfection and changed every 24 h. As shown in Fig. 6, after 48 h of culture AML12 cells transfected with LAT1 demonstrated approximately a 2-fold increase in cell number *versus* GFP-transfected cells after culture without arginine.

To attempt to elucidate the mechanism for this difference in cell number, we performed cell cycle analysis on these cells. AML12 cells transfected with either pFLAG-LAT1 or pEGFP-F were stained with propidium iodide, and cell cycle analysis was performed at 0, 24, and 48 h post-transfection/switch to CEM without arginine. The results are presented in Fig. 7. There were no apparent differences in cell cycle phase distribution after propidium iodide stain in either cell population at any time examined. The observed differences in cell number there-

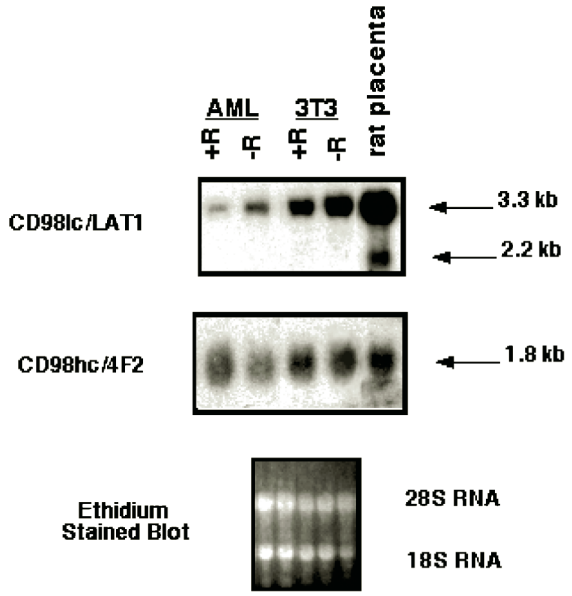

FIG. 5. **Expression of CD98lc/LAT1 and CD98hc/4F2hc in AML12 and NIH3T3 cells in response to arginine availability.** Total RNA was isolated from AML12 and NIH3T3 cells was cultured in CEM with or without arginine for 24 h. Northern blot hybridization of total RNA (12 μg) was analyzed by sequential hybridization to probes for LAT1 (*top panel*) and 4F2hc (*middle panel*). Ethidium bromide staining was used as a loading control between lanes. Approximate transcript sizes are indicated to the *right*. LAT1 but not 4F2hc was induced by arginine deprivation in AML12 but not in NIH3T3 cells. 4F2hc was constitutively expressed in both cell types. LAT1 was constitutively expressed at high levels in NIH3T3 cells relative to AML12 cells.

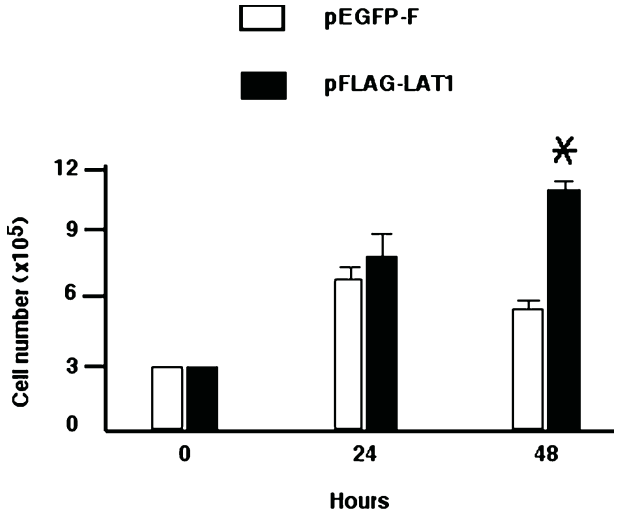

FIG. 6. **Proliferation of LAT1 and GFP transfected AML12 cells cultured without arginine.** AML12 cells were transfected with either pFLAG-LAT1 or pEGFP-F. The cell culture medium was changed to CEM without arginine immediately after transfection. Cells were counted by trypan blue dye exclusion at the beginning of the experiment as well as 24 and 48 h post-transfection/switch to medium without arginine. At 48 h after culture without arginine, the population of LAT1-transfected cells was approximately 2-fold greater ($p < 0.005$) in cell number than the GFP-transfected (control) cells.

fore may be due to differences in rate of proliferation, saturation densities, cell adhesion, or cell survival.

DISCUSSION

We examined the functional significance of overexpression of LAT1/CD98lc and/or 4F2hc/CD98hc in nontransformed hepatic and fibroblast cell lines and found that overexpression of LAT1 alone was sufficient to increase system L transport activity in

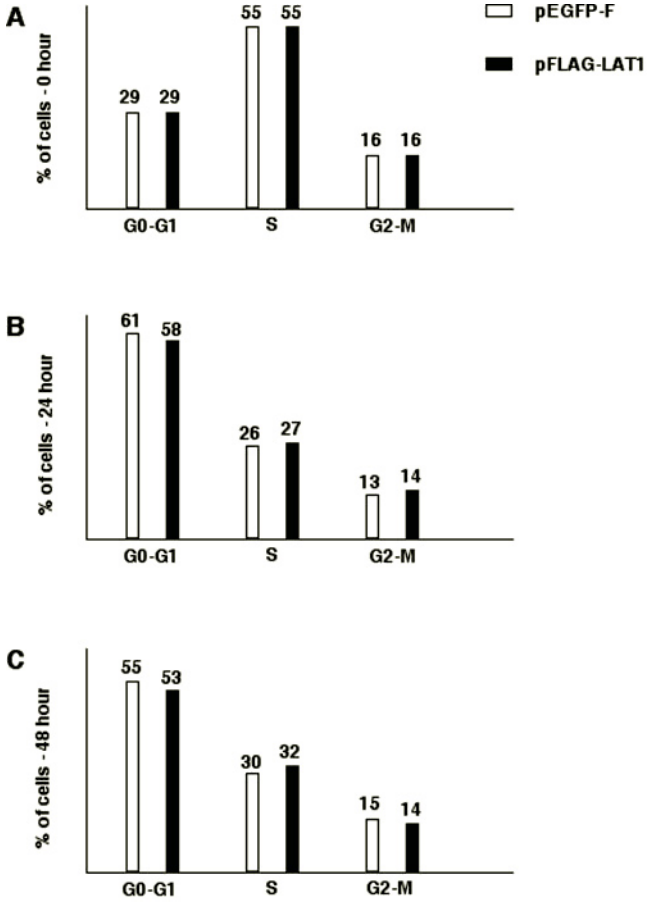

FIG. 7. **Cell cycle analysis in transfected AML12 cells after culture without arginine.** AML12 cells were transfected with either pFLAG-LAT1 or pEGFP-F. The cell culture medium was changed to CEM without arginine immediately after transfection. The cells were stained with propidium iodide and cell cycle analysis was performed at 0 (*A*), 24 (*B*), and 48 (*C*) h post-transfection/switch to CEM without arginine. The percentage of cells in each phase of the cell cycle is shown for each condition. There were no significant differences in cell cycle phase distribution after propidium iodide stain in either cell population at any time examined.

mouse hepatocyte but not fibroblast cell lines. Coexpression of LAT1 and 4F2hc in mouse hepatocytes does not increase system L transport activity above that of LAT1 transfection alone. To our knowledge, this is the first demonstration of increased transport after overexpression of a CD98 light chain in the absence of heavy chain. Exogenous LAT1 presumably complexes with endogenous 4F2hc to mediate transport. Northern analysis and immunofluorescence suggest that 4F2hc message and protein are constitutively expressed at nearly equivalent levels in both mouse hepatocyte and fibroblast cell lines. Endogenous steady-state LAT1 message and protein levels, however, are much higher in mouse fibroblasts *versus* hepatocytes. Thus, the ratio of LAT1/4F2hc seems quite different in these cell types. Because NIH3T3 cells express high levels of both LAT1 and 4F2hc, this suggests that parallel overexpression of these molecules is necessary to elevate transport still higher. In AML12 cells by contrast, endogenous 4F2hc levels are high but LAT1 levels low, and overexpression of LAT1 alone is sufficient to increase transport in AML12 cells.

LAT1 message, but not that of 4F2hc, is responsive to arginine availability in mouse hepatocytes yet not in fibroblasts. We also found that basal amino acid transport through both systems L and y^+ was significantly greater in the fibroblast *versus* hepatocyte cell lines. The increased transport activity in NIH3T3 cells may be necessary to support the much higher

growth rate of NIH3T3 *versus* AML12 cells.[2] Our previous studies have shown that the response of LAT1 and system L transport to arginine availability is lost gradually in transformed and tumorigenic hepatic cells. Our results, therefore, may explain the striking results of Shishido *et al.* (27), in which only a 2-fold increase in 4F2hc expression was sufficient to transform BALB3T3 cells. They suggest a threshold of CD98-mediated malignant transformation, and our results are consistent with the notion that NIH3T3 cells may be close to that threshold. LAT1 response to arginine in the mouse fibroblasts, although not hepatic cells, is similar to our observations in transformed, tumorigenic hepatic cells.

In addition to an increase in system L transport, AML12 cells overexpressing LAT1 also displayed an increase in growth under conditions of limited arginine relative to control cells. An enhanced requirement for amino acids has been identified in the nutrient-poor tumor microenvironment (39). The observed difference in proliferation is a potential mechanism whereby LAT1 expression gives neoplastic cells a growth advantage. Because we did not see any difference in cell cycle phase distribution in any of the cells at any time during the proliferation assay, the observed differences in cell number may be caused by differences in rate of proliferation, saturation densities, cell adhesion, or cell survival. Further studies with stably expressing lines or inducible expression constructs are needed to explore the mechanism(s) involved.

Many factors contribute to neoplastic transformation and malignant progression including expression of oncogenes, loss of tumor suppressors, modulation of angiogenic and cell adhesion factors, loss of cell cycle checkpoint control, etc. There is a growing body of evidence, however, supporting changes in amino acid transport as also being a factor in transformation and carcinogenesis, particularly in the liver. Transport systems L, y^+, ASC, and A show marked differences in activity in normal hepatocytes *versus* hepatomas (25, 40–43). Changes in hepatic amino acid metabolism during carcinogenesis include increases in arginine and glutamine transport in hepatomas *versus* normal hepatocytes and increases in total protein synthesis, proportional to tumor burden, in the livers of tumor-bearing animals (44, 45). Tumor-bearing rats, in contrast to pair-fed controls, experience an increase in hepatic amino acid transport via multiple transport systems, perhaps through an increase in the number of transporter proteins in the plasma membrane (39). It is possible that the failure to observe increased transport in AML12 cells with LAT1 and 4F2hc coexpression relative to LAT1 alone may be caused by a mechanism limiting the number of transporters in the membrane of these normal epithelial cells.

Inhibitors of cellular proliferation have been used to link the increased amino acid transport of hepatomas to the increased proliferation rate of these neoplastic cells. Sodium butyrate and novobiocin inhibited cellular proliferation with a corresponding decrease in leucine, arginine, glutamine, and alanine transport of more than 50% in fast growing SK-Hep cells in 48 h and of 30 and 50% in slow growing HepG2 and Huh-7 cells, respectively (44). The more pronounced inhibitor effect on the faster growing hepatoma is consistent with greater amino acid transport requirements for faster growing cells. Changes in amino acid transport may give neoplastic cells an advantage in metastasizing to new locations as well as an advantage in establishing a primary tumor. The increased transport activity of systems A and N in human adenocarcinoma cells, for example, has been found to enhance the invasiveness of these cells as measured by an amnion and Amgel invasion assay (46).

[2] (W. A. Campbell and N. L. Thompson, unpublished observations)

We have hypothesized that LAT1 expression is an early event in hepatocarcinogenesis providing neoplastic cells with a selective growth or survival advantage, particularly under conditions of nutrient stress. Our data show that overexpression of LAT1 in nontransformed hepatocytes can increase amino acid transport and lead to a growth advantage in conditions of limited amino acid availability. Our data also suggest that the phenotypic consequences of LAT1 expression can vary in different cell types. Cells in which LAT1 is not expressed normally, such as hepatocytes, may acquire a growth advantage if LAT1 becomes constitutively expressed. Studies to examine the mechanisms contributing to constitutive expression and the phenotypic consequences of blocked expression in transformed cells both *in vitro* and *in vivo* are planned to establish the relative contribution of this pathway to tumor growth.

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