

# TA1/LAT-1/CD98 Light Chain and System L Activity, but Not 4F2/CD98 Heavy Chain, Respond to Arginine Availability in Rat Hepatic Cells

LOSS OF RESPONSE IN TUMOR CELLS\*

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**Tumor associated gene-1/L amino acid transporter-1 (TA1/LAT-1) was recently identified as a light chain of the CD98 amino acid transporter and cellular activation marker. Our previous studies with primary rat hepatocyte cultures demonstrated that TA1 RNA levels were responsive to media amino acid concentrations, suggesting adaptive regulation. High level TA1 expression associated with transformed cells also suggested a role in tumor progression. The present study examined the relationship of TA1/CD98 expression, adaptive response, and associated amino acid transport to neoplastic transformation using a panel of well characterized rat hepatic cell lines. We found 1) increased expression of TA1 in response to amino acid depletion, specific for arginine but not glutamine; 2) loss of TA1 response to arginine in  $\gamma$ -glutamyl transpeptidase-positive transformed and tumorigenic cells; 3) no appreciable response of 4F2/CD98 heavy chain to arginine levels; and 4) correlation of system L amino acid transport activity in response to arginine with changes in TA1/LAT-1 mRNA but not total immunoreacting protein. Our results suggest this CD98 light chain may act as an environmental sensor, responding to amino acid availability and that its regulation is complex. We hypothesize that altered TA1 expression is an early event in hepatocarcinogenesis giving neoplastic cells a growth or survival advantage, particularly under conditions of limited amino acid availability.**

TA1<sup>1</sup> was cloned in our laboratory on the basis of its differential expression between rat hepatoma cells and normal adult rat liver and encodes a predicted integral membrane protein with several membrane-spanning domains (1). TA1 is identical

to the C terminus (amino acids 272–512) of LAT-1, which is a 512-amino acid protein with 12 transmembrane domains (2). E16, cloned as a lymphocyte activation antigen, is the human homolog of TA1 and is 95% identical at the amino acid level (3). TA1/LAT-1 is a member of an emerging family of highly conserved molecules with homologs in *Xenopus*, *Schistosoma*, mouse, and man. More recently, TA1/LAT-1 and its homologs have been identified as the light chain of the CD98 molecule (2, 4–8). The CD98 complex consists of an 80-kDa heavy chain (4F2) and a 40–45-kDa light chain (9). CD98 has been implicated in a variety of functions including amino acid transport, cell survival, integrin activation, and cell fusion (10–13). Additional light chains have been identified and designated y<sup>+</sup>LAT-1 (14–16), y<sup>+</sup>LAT-2 (14, 17), and LAT-2 (18, 19). The CD98 complex can mediate System L or System y<sup>+</sup>L amino acid transport in *Xenopus* oocytes depending on which CD98 light chain (CD98lc) is associated with the CD98 heavy chain (CD98hc) (2, 6–8, 14). Co-injection of cRNA for TA1/LAT-1 and 4F2 has been shown to mediate System L transport of large neutral amino acids with branched or aromatic side chains in *Xenopus* oocytes (2, 6). LAT-2 also mediates System L transport in *Xenopus* oocytes upon co-injection with 4F2hc; however, LAT-2 transports both large and small neutral amino acids with a lower affinity ( $K_m$ ) than LAT-1 (18, 19). TA1/LAT-1 is not only expressed in rat hepatomas but also in a variety of human cancers including human colon and breast carcinoma (20). Whereas TA1 expression is not seen in normal adult rat liver, it can be induced transiently in liver and with the same kinetics as *c-myc* after acute carbon tetrachloride injury, suggesting a role in normal injury response in this organ (21). In primary hepatocyte cultures, we also observed that changes in amino acid concentrations resulted in altered levels of TA1 expression, suggesting adaptive regulation (22).

We are interested in examining the regulation and role of TA1/E16 and CD98-related molecules in hepatocarcinogenesis. We have utilized a panel of rat hepatic WB cell lines that differ in their transformed and tumorigenic properties. We have assessed how basal TA1/CD98lc and 4F2/CD98hc expression in response to an environmental factor, arginine availability, are associated with transformation and tumorigenicity in this system. We have found regulation at the level of TA1/CD98lc. The possible physiological significance of this regulation is discussed. 4F2/CD98hc RNA levels remained fairly constant regardless of arginine availability, transformation, or tumorigenicity. TA1/CD98lc RNA levels, but not the total pool of immunologically reactive protein, were found to be modulated by arginine availability in nontransformed and GGT-negative

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<sup>1</sup> The abbreviations used are: TA1, tumor associated gene-1; LAT, L amino acid transporter; 4F2, heavy chain of CD98 cell surface antigen; GGT,  $\gamma$ -glutamyl transpeptidase; KRP, Krebs-Ringer phosphate buffer; FBS, fetal bovine serum; CEM, Chee's essential medium; bp, base pair(s); kbp, kilobase pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TABLE I  
Characteristics of cell lines used in this study

Description of cell lines used in this study as: 1) either positive or negative for transformation (defined as treatment with carcinogen and ability to form a tumor in a subcutaneous site); 2) GGT expression; 3) derivation from an *in vivo* tumor source; 4) high, low, or negative for tumorigenicity at a subcutaneous site or in the liver; and 5) positive or negative for derivation from WB parental rat cell line.

Characteristic	WB	GN6	GN6TF	GP6	GP6TB	GP7TB	1683
Transformed	-	+	+	+	+	+	+
GGT	-	-	-	+	+	+	?
<i>In vivo</i> source	-	-	+	-	+	+	+
Tumorigenicity (subQ)	-	+	+	+	+	+	++
Tumorigenicity (liver)	-	-	-	-	+	+	++
WB-derived	+	+	+	+	+	+	-

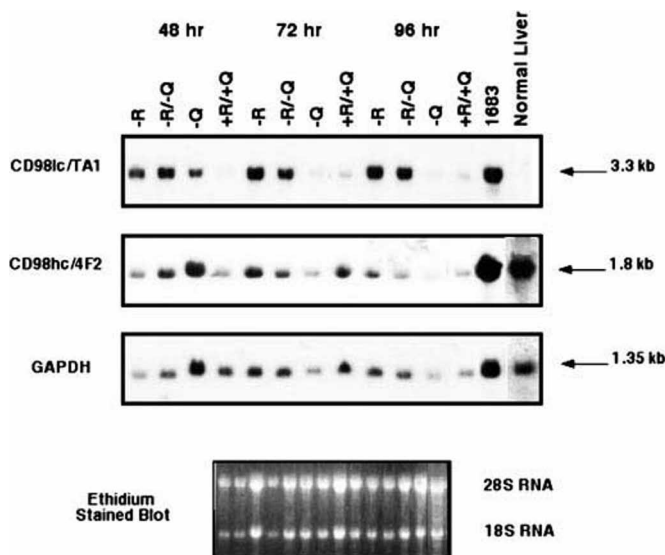


FIG. 1. CD98lc/TA1 and CD98hc/4F2 response to amino acid availability in WB cells. Shown is total RNA from WB cells cultured in CEM without arginine (-R), without arginine and glutamine (-R/-Q), without glutamine (-Q), or CEM with arginine and glutamine (+R/+Q) for 48, 72, and 96 h. Aliquots (12  $\mu$ g) of total RNA were analyzed by sequential Northern blot hybridization to a 900-bp TA1 probe (top panel), a 1.8-kbp 4F2 probe (middle panel), and a 1.25-kbp GAPDH probe (bottom panel). RNA from 1683 rat hepatoma cells and normal adult rat liver were included for comparison. Ethidium bromide staining was used as a loading control between lanes. Approximate transcript size is indicated to the right. Arginine, but not glutamine, availability modulated TA1 RNA levels, whereas neither arginine nor glutamine availability changed 4F2 RNA levels.

transformed WB cells. TA1/CD98lc RNA levels were constitutively high in GGT-positive transformed cells and hepatomas, and TA1/CD98lc protein levels were elevated relative to non-transformed cells. System L amino acid transport was significantly higher in GGT-positive transformed, tumorigenic cells than nontransformed cells and responded to availability of arginine, a nonsystem L substrate.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—A brief description of all cell lines used in this study including data on transformation and tumorigenicity is presented in Table I. The normal diploid hepatic epithelial line WB-F344 (WB) was isolated from an adult male Fischer 344 rat (23). The derivative cell lines were produced by 11 brief repeated exposures of WB-F344 cells to 5  $\mu$ g/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and from this heterogeneous, tumorigenic cell population, 18 clonal subpopulations were isolated based on their expression of the oncofetal marker enzyme GGT (24). Cloned cell lines were determined to be either GGT-negative (*i.e.* GN6) or GGT-positive (*i.e.* GP6). Cell lines were also re-established from subcutaneous tumors derived from GGT-positive (*i.e.* GP6TB, GP7TB) and GGT-negative cells (*i.e.* GN6TF). 1683 cells are rat transplantable hepatocellular carcinoma cells derived from primary carcinomas induced by a choline-deficient, ethionine diet (25).

WB, GN6, GN6TF, and GP7TB cell lines were routinely cultured in

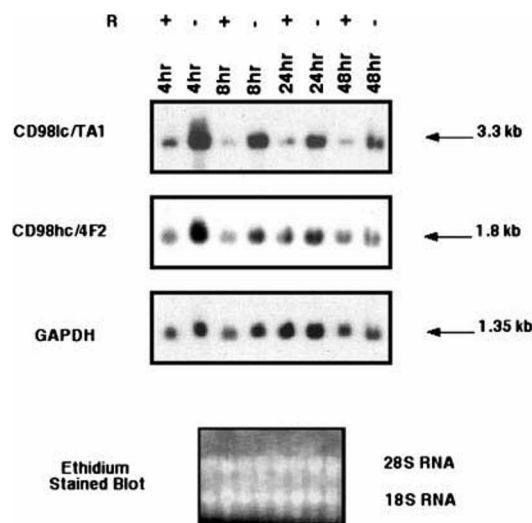


FIG. 2. Time course of CD98lc/TA1 and CD98hc/4F2 response to arginine availability in WB cells. Shown is total RNA from WB cells cultured with arginine (+R) or without arginine (-R) for 4, 8, 24, and 48 h. Aliquots (12  $\mu$ g) of total RNA were analyzed by sequential Northern blot hybridization to a 900-bp TA1 probe (top panel), a 1.8-kbp 4F2 probe (middle panel), and a 1.25-kbp GAPDH probe (bottom panel). Ethidium bromide staining was used as a loading control between lanes. Approximate transcript size is indicated to the right. Steady state TA1 mRNA levels increased within four hours of culture without arginine versus with arginine. Although TA1 mRNA was induced up to ten-fold in WB cells cultured without arginine, 4F2 mRNA levels were induced only 2–3-fold in cells cultured without arginine after densitometric analysis with normalization to GAPDH mRNA levels.

Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% FBS, whereas GP6 and GP6TB were routinely cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) (Life Technologies, Inc.) containing 10% FBS and supplemented with insulin, transferrin, selenous acid, bovine serum albumin, and linoleic acid at 1 ml/100 ml medium (Becton Dickinson). 1683 cells were routinely cultured in Waymouth's medium containing 10% FBS. To better simulate the normal hepatic milieu, a custom formulation of Chee's essential medium (CEM) demonstrated to maintain long term differentiated hepatic cell function *in vitro* was kindly provided by Dr. Hugo Jauregui (Department of Pathology, Rhode Island Hospital) and prepared as described previously (26). Arginine and glutamine were not present in this custom formulation and were added from stock solutions. It was not feasible to selectively delete other amino acids with this medium because custom batches missing other amino acids were only available in 100-liter batches. 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% FBS dialyzed or undialyzed with arginine or without arginine. All experiments with all the cell lines were repeated with dialyzed serum to remove serum as a source of arginine with similar results to nondialyzed serum. Medium was changed every day. Viability was at least 90% in all cases.

**RNA Preparation and Northern Blot Analysis**—Total RNA was isolated using the guanidinium isothiocyanate/cesium chloride method (27) for normal adult rat liver and a modification of that method for cultured cell lines (Totally RNA Kit<sup>®</sup>; Ambion, Austin, TX). RNA was isolated from cultured cells after subculture in CEM at the appropriate

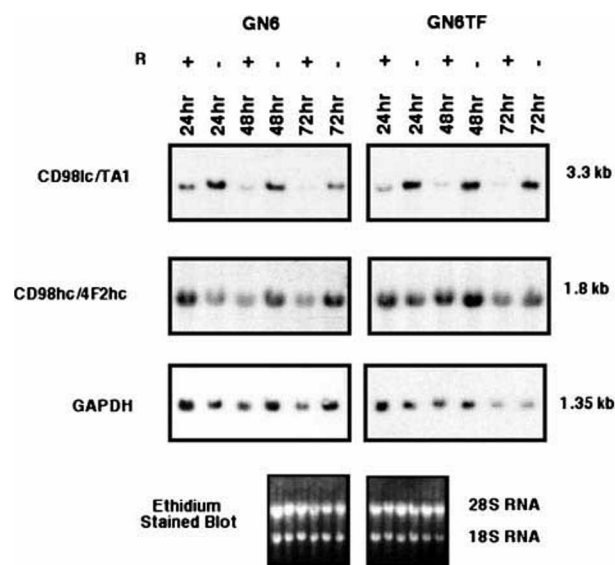
time points. Aliquots (12  $\mu$ g) of total RNA were size fractionated on 1% agarose/formaldehyde gels as described previously (20). After electrophoresis, gels were equilibrated in 1 M ammonium acetate, and RNA was transferred to Nytran nylon membranes (Schleicher & Schuell). Blots were baked for 2 h at 80 °C and hybridized at 65 °C according to Church and Gilbert (28). 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 [<sup>32</sup>P]dCTP (NEN Life Science Products; 3000 Ci/mmol) 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-bp *Eco*RI fragment of human *4F2hc* (provided by Dr. Martin Hemler) (29). Blots were also stripped and hybridized to an 800-bp fragment of the 5' end of LAT-1 derived through reverse transcription-polymerase chain reaction of the 1600-bp coding region of LAT-1 using rat placenta as an RNA source followed by polymerase chain reaction of the 5' end of LAT-1. A 1.25-kbp *Pst*I fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in combination with ethidium bromide staining to evaluate RNA loading variations (30). Densitometry using the Quantity One<sup>®</sup> 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.* (31), and described by Kilberg (32). All <sup>3</sup>H-labeled amino acids were purchased from NEN Life Science Products, and unlabeled amino acids were purchased from Sigma. Cells were seeded in 24-well trays such that the cells would be near confluent for the transport assay. Before the transport assays, cells were rinsed with warm Na<sup>+</sup>-free Krebs-Ringer phosphate buffer (cholKRP), in which the sodium containing salts were iso-osmotically replaced with choline, to remove extracellular Na<sup>+</sup> and amino acids. Cells were incubated in warm cholKRP for 10 min to deplete intracellular amino acids. The uptake of radiolabeled amino acids (5  $\mu$ Ci of <sup>3</sup>H-amino acid/ml) at 100  $\mu$ mol/liter in either 200  $\mu$ l of cholKRP or NaKRP was measured for 30 s at 37 °C. Preliminary experiments indicated that uptake of each <sup>3</sup>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 amino acid 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 cholKRP. 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 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 expressed as pmol amino acid transported per milligram of protein per minute (averages  $\pm$  S.E. of at least three separate determinations). Data comparing two experimental results were analyzed statistically by Student's *t* test using the InStat Macintosh statistics program. Each experiment was repeated at least twice to show qualitatively the same results.

**Immunoblot Analysis**—Detergent extracts were prepared from WB, GP6, and GP7TB cells cultured under the same conditions as for RNA preparation and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (20). Rabbit antibodies prepared to synthetic peptides corresponding to amino acids 211–221 of TA1/E16 (482–492 of LAT-1) deduced sequence were used together with chemiluminescence detection as described previously (20). Two rabbits (K and G) were immunized with the identical preparation of keyhole limpet hemocyanin-conjugated peptide. A recombinant fusion protein of TA1 with six histidines produced in baculovirus and purified by nickel affinity chromatography as previously reported (4) served as positive control. As a negative control, blots were reacted with nonimmune rabbit serum at the same dilution.

## RESULTS

**TA1 mRNA Is Induced Following Arginine Deprivation of WB Cells While 4F2hc mRNA Levels Remain Invariant**—In previous studies with primary hepatocyte cultures, we found that TA1 mRNA levels could be either induced or suppressed depending on the culture medium and its amino acid concentrations (22). We extended these studies to examine whether



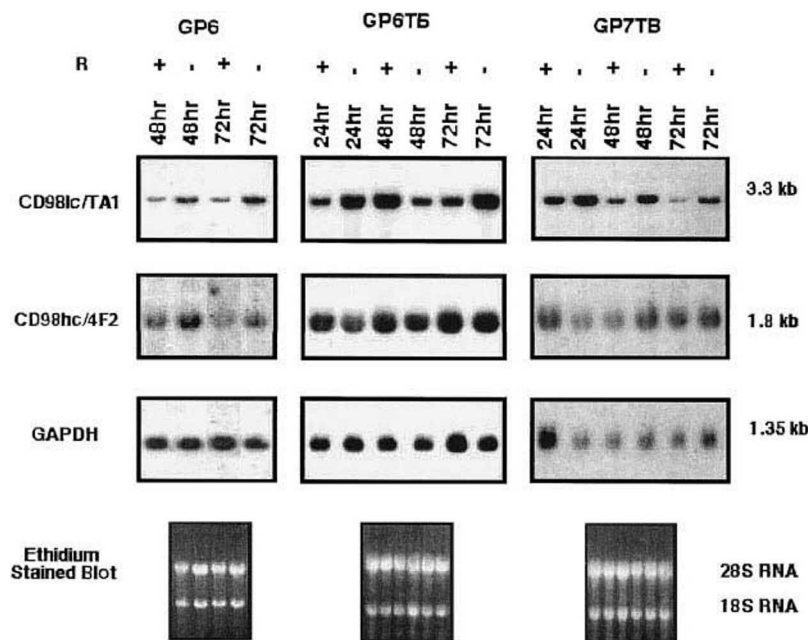
**FIG. 3. Expression of CD98hc/TA1 and CD98hc/4F2hc in cultured GN6 and GN6TF cells in response to arginine availability.** Shown is total RNA from GN6 and GN6TF cells cultured in CEM with or without arginine for 24, 48, and 72 h. Aliquots (12  $\mu$ g) of total RNA were analyzed by Northern blot hybridization to a 900-bp TA1 probe (*top panel*), stripped and then rehybridized with a 1.8-kbp 4F2hc probe (*middle panel*), or stripped and then rehybridized with a 1.25-kbp GAPDH probe (*bottom panel*). Ethidium bromide staining was used as a loading control between lanes. Approximate transcript sizes are indicated to the right. Steady state TA1 RNA levels were high in cells cultured without arginine but were gradually lowered in cells cultured with arginine. 4F2hc RNA levels were constitutively high.

the same response to amino acid availability was found in a normal rat liver epithelial cell line, WB, and whether deprivation of arginine and/or glutamine, the two amino acids manipulable in our culture system, could increase TA1 expression in these cells. WB is a diploid cell line isolated from the liver of an adult male Fischer-344 rat and has been used extensively as both an *in vitro* and an *in vivo* model system for the liver (23, 33). WB cells were cultured in CEM, CEM without arginine, CEM without glutamine, or CEM without arginine or glutamine for 48, 72, and 96 h. Using densitometry and normalizing to GAPDH, steady state levels of TA1 RNA were 8–10-fold higher in WB cells cultured in the absence of arginine than in the presence of arginine (Fig. 1). Thus, the availability of arginine was found to modulate TA1 expression, whereas glutamine availability had no effect. The apparent increase in TA1 observed after WB cells are cultured for 48 h without glutamine was found to be negligible after standardization to ethidium staining and GAPDH hybridization. In stark contrast to TA1, 4F2hc mRNA levels did not vary in response to arginine availability. Similarly, glutamine availability did not affect 4F2hc expression. Other amino acids were not examined because of constraints of medium composition. Since arginine availability was found to modulate TA1 mRNA levels in these nontransformed nontumorigenic cells, we used arginine availability subsequently as a tool to examine the regulation of TA1 and 4F2hc in a panel of rat hepatic cell lines differing in stages of transformation and tumorigenicity.

**Early Response of TA1 and 4F2hc mRNA to Arginine Availability**—To determine the time course of the observed response, we examined TA1 and 4F2hc steady state message levels at additional time points in WB cells cultured with and without arginine. WB cells were cultured in Dulbecco's modified Eagle's medium, trypsinized and then seeded and cultured in CEM with or without arginine for 4, 8, 24, and 48 h. The results are presented in Fig. 2. TA1 steady state mRNA levels were responsive to arginine availability in WB cells within four hours



FIG. 4. Expression of CD98lc/TA1 and CD98hc/4F2 in cultured GP6, GP6TB, and GP7TB cells in response to arginine availability. Shown is total RNA from GP6, GP6TB, and GP7TB cells cultured in CEM with or without arginine for 24, 48, and 72 h (Note: RNA for GP6 cells at 24 h was not available). Northern blot hybridization of total RNA (12  $\mu$ g) was analyzed by sequential hybridization to probes for TA1 (top panel), 4F2hc (middle panel), and GAPDH (bottom panel). Ethidium bromide staining was used as a loading control between lanes. Approximate transcript sizes are indicated to the right. Neither TA1 nor 4F2 RNA levels varied significantly in any cell line (more than 2–3-fold) under these culture conditions.



after culture in CEM without arginine. After densitometric normalization to GAPDH steady state mRNA levels, TA1 mRNA levels were approximately ten-fold greater in cells cultured without arginine for four hours *versus* with arginine. Normalized 4F2 mRNA levels at this time point were approximately 2.5 fold higher in WB cells cultured without arginine *versus* with arginine. For subsequent experiments, we chose to examine differences between response of normal and transformed, tumorigenic cell lines at time points from 24 to 72 h, since cell attachment and spreading of this integrin-associated molecule may confound data interpretation.

**TA1 and 4F2 RNA Levels Differentially Respond to Arginine Availability in GGT-negative Transformed WB Cells versus GGT-positive Transformed WB Cells**—Loss of response to positive/negative signals is one of the major events marking the transition from a normal to a malignant cell. We examined TA1 and 4F2 response to arginine availability in chemically transformed GGT-negative (GN6) and GGT-positive (GP6) WB cells, and tumor derived WB cell lines which were both GGT-negative (GN6TF) and GGT-positive (GP6TB, GP7TB) to determine if TA1 and/or 4F2 response would correlate with transformation, GGT status, or tumorigenicity. Normally, GGT is expressed in fetal rat hepatocytes, adult bile duct cells, and kidney, but during hepatocarcinogenesis it becomes up-regulated as an early event in neoplastic conversion. Hanigan has elegantly demonstrated that GGT-negative and GGT-positive mouse hepatoma (Hepa 1–6) cells grow similarly in cysteine-rich medium (>100  $\mu$ M cysteine), but at physiological concentrations of cysteine (<100  $\mu$ M cysteine), GGT expression confers a selective growth advantage on GGT-positive mouse hepatoma cells (34). Cells were cultured in CEM with or without arginine for 24, 48, and 72 h prior to RNA extraction. Northern analysis was performed and the results are shown in Figs. 3 and 4. Although 4F2 expression remained fairly constant in all cells, conditions, and time points, TA1 expression varied considerably. In the GGT-negative WB lines, TA1 RNA levels increased 8 to 10 fold in the absence of arginine for 72 h. In contrast, expression increased only about 2 to 4 fold in GGT-positive transformed cells largely because of higher basal level expression in the presence of arginine. Similar results were also obtained at the earlier time points, although the fold differences were lower.

Despite the transformation status of these cell lines, in all cases TA1 RNA was expressed at a high level in cells cultured without arginine. Interestingly, although basal 4F2 expression was about 2-fold greater in transformed cells, it remained fairly constant for each cell type whether or not arginine was present in the medium. At early time points after culture in CEM, GGT-negative transformed cells express high levels of TA1 regardless of arginine availability, similar to GGT-positive cells at all time points. After 72 h of culture in the presence of arginine, reduced TA1 levels were observed in GGT-negative transformed cells similar to those seen in nontransformed WB cells cultured in CEM with arginine. These results imply the loss of regulation of TA1 at different stages of progression in these rat hepatic lines.

**TA1 and 4F2 Are Constitutively Expressed in a Non-WB Rat Hepatoma Cell Line**—We also examined the response of TA1 and 4F2 in another rat hepatoma cell line separate from the WB tumor-derived cell lines. In contrast to WB lines that were transformed by carcinogen treatment *in vitro*, 1683 cells are rat transplantable hepatocellular carcinoma cells derived from primary carcinomas induced by feeding rats a choline-deficient, ethionine diet (25). 1683 cells were cultured in CEM with or without arginine for 24, 48, and 72 h. Neither TA1 nor 4F2 expression significantly changed whether or not arginine was included in the medium (Fig. 5). Steady state levels of both messages were high all the time, with only slight increases of TA1 expression (2–3-fold) at 72 h after arginine deprivation. These cells constitutively express high levels of TA1/CD98lc and 4F2/CD98hc message. Thus loss of arginine response does not appear related to the type of carcinogen involved in transformation.

**Relative TA1 and 4F2 mRNA Levels in All Cell Lines Examined**—RNA samples from each cell line were included on the same Northern blot so that comparisons could be made between all the cell lines. These results are presented in a graph in Fig. 6. Each bar represents the average densitometric value determined from three independent blots. 4F2/CD98hc RNA levels are fairly constant in every cell line, and every time point examined had no greater than a 2-fold difference between cells cultured with or without arginine. WB cells have the lowest TA1 and 4F2 RNA levels in all conditions and time points, although TA1 RNA levels do increase 8–10-fold when cells are

cultured without arginine. When GN6 and GN6TF cells were cultured in medium with arginine, TA1 RNA levels showed a large decrease from 24 to 72 h relative to cells maintained without arginine. It is also interesting to compare the TA1 response to arginine availability by *in vitro* transformed cells (GP6 cells) versus cell cultures derived from solid tumors of these cells (GP6TB). In both GP6 and GP6TB, TA1 RNA levels differ only about 2–4-fold during the course of culture with or without arginine. GP6TB cells, however, express higher basal levels of both TA1 and 4F2 than GP6 cells and also induce higher levels of TA1 when cells are cultured without arginine, suggesting an alteration associated with tumor progression.

**System L Transport Is Differentially Regulated in Nontransformed Versus GGT-positive Transformed and Tumorigenic Rat Hepatic Cells**—Because TA1/LAT-1 functions as the light chain of CD98 and has been demonstrated to mediate system L transport in *Xenopus* oocytes, we hypothesized that the altered expression of TA1 was likely to be associated with differences in transport activity. We thus examined whether arginine availability affected system L amino acid transport in WB cells and whether there were differences between nontransformed and transformed GGT-positive tumorigenic lines. As shown in Fig. 7a, WB cells cultured without arginine for 48 h transported approximately 3-fold more leucine (significant at  $p < 0.05$ ) than WB cells cultured in the presence of arginine regardless of the presence of sodium. As expected, addition of excess cold tyrosine inhibited leucine transport, consistent with system L activity. Arginine transport (system  $y^+$ ) was not significantly different in WB cells cultured with or without arginine (Fig. 7b). As shown in Fig. 7c, GP7TB cells cultured without arginine for 48 h also transported significantly more leucine (about 2.5-fold) than GP7TB cells cultured with arginine. Arginine transport was not significantly different in GP7TB cells under the two culture conditions (Fig. 7d).

GP7TB cells transported approximately 50% more leucine than WB cells whether the cells were cultured with arginine or without arginine (significant at  $p < 0.05$ ). These data correlate well with TA1 mRNA levels in GP7TB and WB cells cultured with and without arginine for 48 h (compare Figs. 1 and 4) and are consistent with its role as a system L transporter. The increase in transport seen in these cells is less than the increase in mRNA levels, suggesting that transcriptional mechanisms alone are unlikely to account for regulation.

**TA1 Immunoreactive Protein Pools Are Elevated in Transformed and Tumorigenic Cell Lines**—To determine whether levels of TA1 protein in transformed lines derived from the WB

line were increased relative to normal hepatic cells and how these levels compared with transport activity, immunoblot analysis was performed with anti-TA1 peptide antibodies (Fig. 8). Lanes were loaded with equivalent amounts of total protein per cell extract (15  $\mu$ g), as confirmed by Ponceau S staining after gel transfer (not shown). Relative to nontransformed WB cells, K anti-peptide antibody detected multiple bands in cell extracts from transformed GP6 and tumorigenic GP7TB cells. The size range of these bands is similar to what has been reported for *in vitro* translated 512-amino acid LAT-1 (2). Although antibodies from both rabbits reacted with the recombinant TA1, for reasons that are not known, rabbit serum

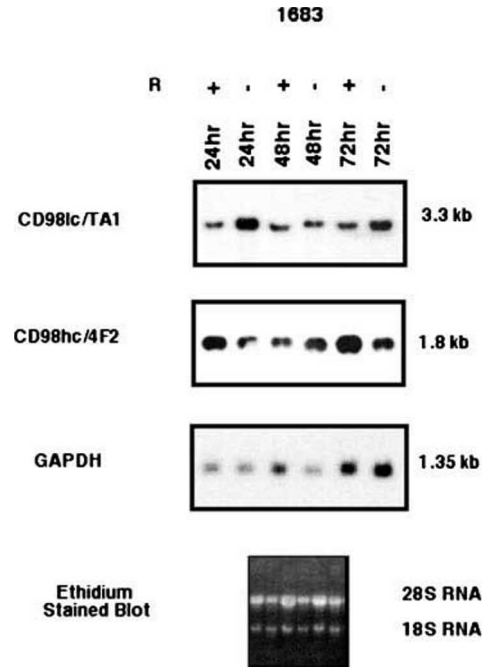
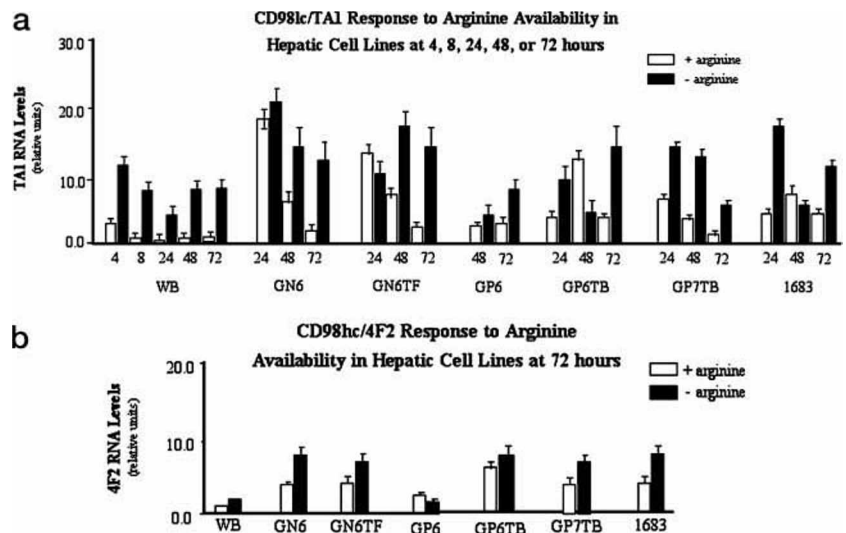


FIG. 5. Expression of CD98lc/TA1 and CD98hc/4F2 in cultured 1683 hepatoma cells in response to arginine availability. Total RNA from 1683 cells cultured in CEM with or without arginine for 24, 48, and 72 h. Northern blot hybridization of total RNA (12  $\mu$ g) was analyzed by sequential hybridization to probes for TA1 (top panel), 4F2hc (middle panel), and GAPDH (bottom panel). Ethidium bromide staining was used as a loading control between lanes. Approximate transcript sizes are indicated to the right. Both TA1 and 4F2 were constitutively expressed at high levels in these tumorigenic cells throughout the time course of the experiment.

FIG. 6. Graphs of relative CD98lc/TA1 and CD98hc/4F2 mRNA levels in the various rat hepatic cell lines. RNA samples from each cell line were included on the same Northern blot to yield these comparisons. Each bar represents the average densitometric value determined from three independent blots. a, relative units were plotted for TA1 mRNA levels in the various cell lines cultured in the presence (open bars) or absence (closed bars) of arginine for 4, 8, 24, 48, or 72 h. b, relative units were plotted for 4F2 mRNA levels in the various cell lines cultured in the presence (open bars) or absence (closed bars) of arginine for 72 h. RNA levels at 72 h are representative of levels at earlier times because there was little change in 4F2 RNA levels.



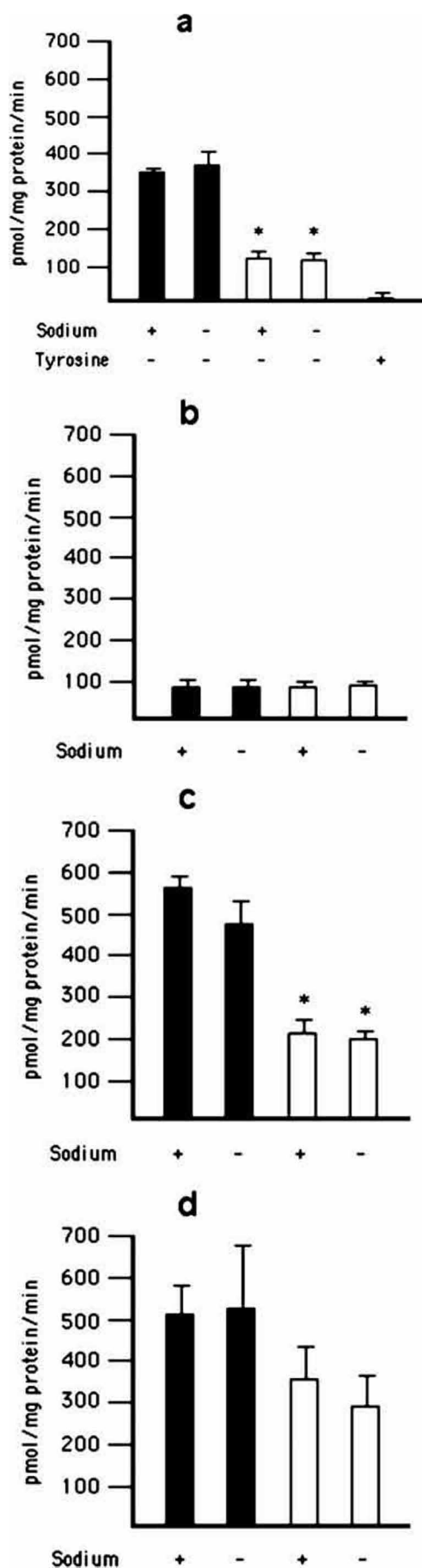


FIG. 7. Amino acid transport activity of leucine and arginine in WB and GP7TB cells. The uptake of 100  $\mu$ M radiolabeled leucine

G reacted only with a band of approximately 35 kDa present in GP7TB cells. In contrast to the increase observed in message levels in cells cultured without arginine, no increase was observed in any immunoreactive bands under these culture conditions. None of the bands shown were present after blots were incubated with the same dilution of nonimmune rabbit serum (data not shown). Whether the multiple bands detected with antibody K may correspond to proteolytic fragments or TA1/LAT-1 cross-reactive molecules is not known.

#### DISCUSSION

We have examined the response of TA1/CD98lc and 4F2/CD98hc expression and amino acid transport to arginine availability in a panel of rat hepatic epithelial cell lines differing in GGT-expression, transformation, and tumorigenicity. We have focused our initial studies primarily at the RNA level to assess changes associated with tumor progression. The CD98 light chain has been implicated by others in amino acid transport specificity (2, 6–8, 14). Our results show that steady state levels of TA1/CD98lc RNA can be up-regulated 10-fold in non-transformed cells in response to arginine but not glutamine deprivation, whereas 4F2hc levels only vary by 2-fold under identical conditions. To our knowledge this is the first demonstration that the light and heavy chains of the CD98 complex respond differentially to an environmental cue. Using this medium formulation, we were not able to deplete other nutrients, and thus it is not known if the response of CD98lc is specific to the stress of this amino acid limitation. However, we have not observed up-regulation after rat hepatic cells are cultured in conditions of heat shock, hypoxia, or anoxia (data not shown). This study was not intended to be a complete study of nutrient response, and further studies are needed to determine the specificity, threshold, and reversibility of the response.

TA1/CD98lc is constitutively expressed in GGT-positive transformed WB (both *in vitro* transformed and tumor-derived) cells and 1683 cells. When comparing the tumor-derived cell lines, it is interesting to note that GGT-negative GN6TF cells, which can modulate TA1 RNA levels in response to amino acid availability, do not form tumors in the liver, whereas the GGT-positive tumor-derived cells (GP6TB and GP7TB), which do not modulate TA1/CD98lc RNA levels, do form tumors rapidly in the liver (33). 4F2 mRNA levels do not vary significantly in these cells, although in general the levels are much higher in transformed *versus* nontransformed cells (Fig. 6b and compare 4F2 mRNA levels in WB *versus* GN6, GN6TF, GP6TB, GP7TB, and 1683). System L activity was also found to respond to arginine availability and correlated with TA1 mRNA levels in the cells. Tumorigenic rat hepatic cells express higher basal and inducible system L activity than nontumorigenic cells. We have also shown that the loss of response to arginine can occur at various points in hepatic cell transformation/progression. By the time the cells have attained tumorigenic capacity, the regulation is lost.

The correlation between alteration in TA1 regulation and

and arginine was measured in both WB and GP7TB cells cultured in CEM without arginine (*closed bars*) or with arginine (*open bars*) for 48 h. All transport values are expressed as pmol/mg of protein/min. Each experiment was repeated at least twice with similar results. Values are the means  $\pm$  S.E. of three measurements. Values from a representative experiment are shown. *a*, leucine transport was measured in WB cells. Excess cold tyrosine inhibited leucine transport. *b*, arginine transport was measured in WB cells. *c*, leucine transport was measured in GP7TB cells. *d*, arginine transport was measured in GP7TB cells. Leucine transport was as follows: GP7TB-R > WB-R > GP7TB + R > WB + R. These results correlated well with TA1/LAT-1 mRNA levels. Arginine transport did not vary in cells cultured with or without arginine; however, the tumorigenic GP7TB cell line transported more arginine than immortalized but not transformed WB cells.

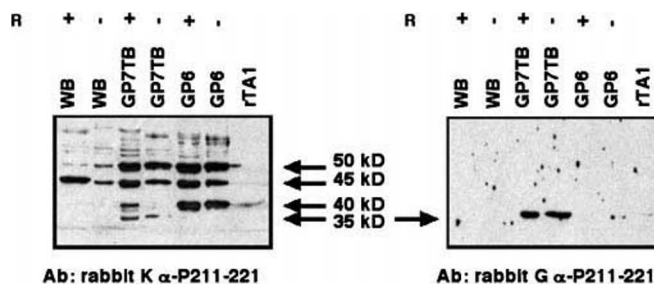


GGT expression is interesting because hepatic GGT is up-regulated during carcinogenesis as an early event in neoplastic conversion (35–37). Hanigan and Ricketts (38) have found that under conditions of low cysteine, such as those found in the tumor microenvironment, GGT converts glutathione to a source of cysteine. Furthermore, GGT expression confers a selective growth advantage on GGT-positive mouse hepatoma cells at physiological concentrations of cysteine (34). Similarly, we hypothesize that TA1 expression may give cells a selective growth or survival advantage particularly under conditions of enhanced requirements for amino acids as is found in the tumor microenvironment (39).

Perhaps not surprisingly, our immunoblot analysis failed to show an alteration in the total pool of immunoreactive TA1/LAT-1 comparable with the observed changes in RNA levels or transport activity, although we did see elevated TA1/LAT-1 protein in transformed and tumorigenic cells relative to non-transformed cells. These studies are complicated by a number of factors including assessment of cell surface *versus* cytoplasmic pools of protein, free light chain *versus* that in complex with heavy chain and whether antibodies cross-react with closely related members of the LAT-1 family, including LAT-2, which may be expressed in these cells. Furthermore, as was recently demonstrated for the arginine transporter, *cat-1*, translation of specific pools of messenger RNAs may be compromised under conditions in which amino acids are limiting (40). Because our light chain anti-peptide antibodies were not able to immunoprecipitate the CD98 complex, further studies involving specific heavy chain antibodies and cell localization or subfractionation will be necessary to fully assess regulation of this molecule.

Hepatic amino acid transport and transporters are known to change dramatically during the process of hepatocarcinogenesis such that transporters up-regulated in neoplastic cells are often different from their counterparts in normal cells (41). There are at least two System L activities in liver: L<sub>1</sub> is a high affinity transporter found in fetal hepatocytes and transformed cells, and L<sub>2</sub> is a low affinity transporter located in freshly isolated hepatocytes (42, 43). LAT-1/TA1 may correspond to the L<sub>1</sub> transporter, whereas LAT-2 or an unidentified light chain may correspond to the L<sub>2</sub> transporter. The present data do not exclude the possibility that TA1 is a partial cDNA of LAT-1 or alternatively that TA1 is a 6-transmembrane variant of the 12-transmembrane form. The detection of an immunoreactive protein of a smaller size than the 40-kDa major band in GP7TB cell extracts by two distinct anti-peptide antibodies is noteworthy in this regard.

Amino acid availability may be one of the signals that regulates the expression of transport proteins, and loss of response to amino acid availability may contribute to the neoplastic process. Some examples of mammalian mRNAs or proteins whose synthesis is enhanced in response to amino acid deprivation include: *cat-1* (40), asparagine synthase (44), ornithine decarboxylase (45, 46), insulin-like growth factor binding protein-1 (47), System A (48) and System L (49) amino acid transport, and *c-jun* and *c-myc* (46, 50). Although modulation of a System L amino acid transporter by a System y<sup>+</sup> substrate may appear contradictory, there are numerous examples in which deprivation of amino acids can lead to increases in expression of genes even though the deprived amino acid is not directly synthesized/transported by the gene product. Examples include asparagine synthetase (44), *jun*, *fos*, and *myc* (46), *cat-1*, System A, and L17 (50, 51). Kilberg *et al.* (51) has hypothesized that mammalian cells may have a general response to amino acid deprivation similar to the response seen in yeast such that deprivation of any single amino acid up-regulates various activities.



**FIG. 8. Immunoblot analysis of CD98lc/TA1 in WB, GP7TB, and GP6 cells.** Detergent extracts from WB, GP7TB, and GP6 cells cultured in CEM with or without arginine for 72 h and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Rabbit antibodies (K and G) prepared to synthetic peptides of CD98lc/TA1 (20) were used together with chemiluminescence detection. A recombinant six-histidine fusion protein of TA1 (*rTA1*) produced in baculovirus (3) served as a positive control. Relative to nontransformed WB cells, K anti-peptide antibody specifically detected multiple bands in cell extracts from transformed GP6 and tumorigenic GP7TB cells. The size range of these bands is similar to what has been reported for *in vitro* translated 512-amino acid LAT-1 (2). The 35-kDa band was detected only in GP7TB cells, and this band was the only band detected by G anti-peptide antibody.

In our current working model, 4F2/CD98hc is proposed to associate with a low affinity transporter such as LAT-2 or an as yet unidentified member of the LAT family in the normal adult hepatocyte. Under conditions of transient nutrient stress, the high affinity transporter TA1/LAT-1 would be up-regulated transiently and serve as the major System L activity associated with 4F2. During hepatic cell transformation/progression to a tumorigenic cell, this regulation would be lost such that high affinity System L transport becomes constitutive.

CD98 has been associated with many functions in different cell types. Up-regulation of TA1 in hepatic cells may conceivably block or uncouple other (*e.g.* nontransport) functions including CD98 clustering and thereby contribute to neoplastic transformation. Recently, Hara *et al.* (52) have shown that overexpression of human 4F2/CD98hc in murine NIH3T3 cells resulted in malignant transformation of these cells. Whether levels of the light chain or heavy to light chain ratios were affected was not examined in this study. Although it is not yet known whether 4F2/CD98hc overexpression would have similar effects in epithelial cells, our studies suggest a potential mechanism whereby CD98lc may contribute to malignant transformation of hepatic cells by providing neoplastic cells with a selective growth advantage, particularly under conditions of nutrient stress. Experiments are underway to test this hypothesis directly.

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