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Evidence for a Regulatory Element Controlling Amino Acid Transport System L in Chinese Hamster Ovary Cells

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Abstract We have used the technique of somatic cell hybridization to study the regulation of the neutral amino acid transport system L in Chinese hamster ovary (CHO) cells. The cell line CHO–*ts*025C1 has a temperature-sensitive mutation in leucyl-tRNA synthetase. At the nonpermissive temperature of 39°C, CHO–*ts*025C1 cells are unable to charge leucyl-tRNA and behave as though starved for leucine by increasing their system L transport activity two- to fourfold. From the temperature-sensitive cell line, we have isolated a regulatory mutant cell line, CHO–C11B6, that has constitutively elevated system L transport activity. The CHO–C11B6 cell line retains the temperature-sensitive leucyl-tRNA synthetase mutation, but growth of this cell line is temperature resistant because its increased system L transport activity leads to increased intracellular leucine levels, which compensate for the defective synthetase. Hybrid cells formed by fusion of the temperature-sensitive CHO–*ts*025C1 cells and the temperature-resistant CHO–C11B6 cells show temperature-sensitive growth and temperature-dependent regulation of leucine transport activity. These data suggest that the system L activity of CHO cells is regulated by a dominant-acting element that is defective or absent in the regulatory mutant CHO–C11B6 cell line.

Key words: amino acid transport, transport regulation, transport system L, CHO cells, hybrid cells

The mammalian cell neutral amino acid transport system L is Na^+-independent and transports mainly branched-chain and aromatic amino acids [Collarini and Oxender, 1987]. System L activity is regulated by amino acid availability in Chinese hamster ovary (CHO) cells. Severe starvation of CHO–K1 cells for leucine causes system L transport activity to increase three- to fourfold [Moreno et al., 1985]. Regulation of system L can also be observed in the CHO cell lines CHO–*ts*H1 and CHO–*ts*025C1, two temperature-sensitive leucyl-tRNA synthetase mutants [Thompson et al., 1973; Giles et al., 1980]. At the nonpermissive temperature of 39°C, these cells are unable to charge leucyl-tRNA and behave as though starved for leucine, and system L transport activity increases two- to fourfold [Moore et al., 1977; Shotwell et al., 1982; Lobaton et al., 1984]. The temperature-dependent enhancement of transport activity in these cell lines is specific to system L and is accompanied primarily by an increase in the V_{max} value of Na^+-independent leucine uptake [Shotwell et al., 1982; Lobaton et al., 1984].

We have isolated temperature-resistant cell lines from mutagen-treated CHO–*ts*H1 cells by selecting for survival at 39°C and low leucine concentrations [Shotwell et al., 1983]. The temperature-resistant cell lines show increased and unregulated system L transport activity compared to the parental cell line, with no changes in the other neutral amino acid transport systems [Shotwell et al., 1983]. These cell lines still retain the temperature-sensitive leucyl-tRNA synthetase [Shotwell et al., 1983]. The temperature resistance of these cell lines appears to be the result of increased and unregulated system L transport activity, which leads to increased intracellular leucine levels. The increased intracellular levels of leucine in the regulatory mutant cell lines compensate for the defective leucyl-tRNA synthetase in essentially the same way

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that higher levels of leucine in the media permit growth of the parental CHO–*ts*H1 cell line at nonpermissive temperatures [Mulnar and Rauth, 1975, 1979; Farber and Deutscher, 1976]. These results suggest that the temperature-resistant regulatory transport mutants may be defective in an element that is necessary for system L regulation [Shotwell et al., 1983].

To further investigate the mechanism of system L regulation, we used the technique of somatic cell hybridization to form CHO cell hybrids between the regulatory mutant cell line CHO–C11B6 and the CHO–*ts*025C1 cell line, the only difference between these two cell lines being the alteration in system L regulation [Shotwell et al., 1983; Lobaton et al., 1984]. This paper presents evidence that system L activity may be controlled by a dominant regulatory element.

MATERIALS AND METHODS

Cell Lines and Culture Methods

The temperature-sensitive leucyl-tRNA synthetase mutant cell line CHO–*ts*025C1 was obtained from R.E. Giles. This cell line is defective in the enzyme hypoxanthine phosphoribosyltransferase ($HPRT^-$) [Giles et al., 1980]. These cells were transfected with the cosmid vector pCV108, which carries the gene coding for resistance to the antibiotic G418 [Lau and Kan, 1983]. The transfected subclone of the CHO–*ts*025C1 cell line used in these studies, referred to as CHO–R100 [El-Gewely and Oxender, 1985], was maintained at 34°C in Eagle's minimal essential medium (MEM) (KC Biological, Lenexa, KS), containing Earle's salts and nonessential amino acids, supplemented with 5% (v/v) fetal calf serum (FCS) (KC Biological), 2.2 g/L sodium bicarbonate, and 200 μg/ml G418 (GIBCO, Grand Island, NY). The cell line CHO–C11B6 was isolated from mutagen-treated CHO–*ts*H1 cells in our laboratory [Shotwell et al., 1983]. This cell line was maintained at 39°C in leucine-free MEM containing Earle's salts and nonessential amino acids, supplemented with 5% (v/v) dialyzed FCS, 2.2 g/L sodium bicarbonate, and L-leucine to a final concentration of 0.1 mM. All cultures were maintained at pH 7.4 in a humidified incubator equilibrated to a 5% CO_2 atmosphere. Cells were harvested using a 0.01% trypsin solution in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, consisting of 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. For transport assays, a sodium-free buffer, pH 7.4, was used in which equimolar amounts of choline chloride and choline phosphate replaced the NaCl and the sodium phosphate, respectively. This buffer is referred to as PBC and was prepared as described previously [Shotwell et al., 1981]. A modification of PBC, with 5.6 mM D-glucose, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$, pH 7.4, was referred to as PBC–GMC.

Hybridizations

Hybridizations were carried out using established procedures [Adair et al., 1978; Klebe and Mancuso, 1981]; 4–5 × 10^5 cells of each cell line were plated together in one well of a 24-well dish (Costar) and incubated overnight at 34°C in MEM with 5% FCS. The next day, the cells were washed with 2 ml minimal attachment medium (MAM), containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, and 10 mM HEPES, pH 7.55. The medium was removed, and 200 μl of 50% (v/v) polyethylene glycol (PEG-1000, Baker, Phillipsburg, NJ) in 0.15 M HEPES, pH 7.55, was added to the cells for 1 min at room temperature. The cells were washed 3 times with 2 ml MAM and incubated for 30 min at 34°C in 2 ml MAM. The cells were then incubated overnight at 34°C in MEM with 5% FCS. The next day, the cells were trypsinized and plated in 100-mm dishes (Corning) at a density of 5 × 10^4 cells/dish in 10 ml MEM with 5% FCS and incubated overnight at 34°C. The next day, the medium was replaced with MEM containing 5% FCS, 500 μg/ml G418, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (using HAT ×50 supplement, Sigma). After 9 days at 34°C, colonies were transferred and maintained in selective medium at 34°C.

Chromosome Counts

Preparation of parental and hybrid cells for chromosome counts followed published procedures [Worton and Duff, 1979].

Amino Acid Transport Assays

The cluster tray assay was used for the transport assays [Gazzola et al., 1981]. Cells were plated at 1.4 × 10^5 (CHO–R100 and CHO–C11B6 cell lines) or 0.7 × 10^4 (hybrid cell lines) in MEM with 5% FCS in a 24-well dish (Costar) and assayed after 16–20 h. The hybrids cells were larger and had higher protein values per cell than the parental cells, so they were plated

at a lower density to ensure that all cell lines were at approximately the same level of confluence. The cells were washed with 2 ml PBC–GMC and incubated for 40 min at 34°C with a change of PBC–GMC at 20 min. We have established that this protocol completely eliminates any possible *trans*-effects [Collarini et al., 1989]. The Na^+-independent uptake of 50 μM leucine was then determined as previously described [Shotwell et al., 1981]. Cellular protein was determined using the method of Zak and Cohen [1961], as described previously [Shotwell et al., 1981]. The data were expressed as nanomoles leucine taken up per milligram protein per min.

Kinetic Analysis

Initial rates of uptake were determined for a series of leucine concentrations. Analyses of kinetic constants were carried out using a PASCAL program applying the Marquardt nonlinear least-squares method to fit the parameters of the following equation:

$$v = (V_{max}[S]/K_m + [S]) + P[S].$$

In this equation, v, S, V_{max}, and K_m have their usual meanings. P is the first-order term describing the nonsaturable uptake.

Preparation of Double-Dialyzed Fetal Calf Serum

Commercially available dialyzed FCS contains small but significant (~10 μM) amounts of leucine [Moreno et al., 1985]. Dialyzed FCS (KC Biological) was further dialyzed against 0.9% NaCl (1:20) for 72 h with two changes of the saline solution.

Plating Efficiency

Cells were plated at approximately 100 cells per 100-mm dish in leucine-free MEM supplemented with 5% (v/v) double-dialyzed FCS and 50 μM leucine. The medium for the hybrid cell lines was also supplemented with HAT and 500 μg/ml G418. The cells were incubated at the indicated temperature for approximately 10 days, then fixed with 95% ethanol and stained with 0.1% crystal violet.

Materials

L-[^{3}H]leucine (50–60 Ci/mmol) was obtained from ICN. L-leucine, trypsin, and crystal violet were purchased from Sigma.

RESULTS

Hybridization

CHO–*ts*025C1 cells, which are $HPRT^-$, have been transfected with the cosmid vector pCV108, which carries the gene coding for resistance to the antibiotic G418 [Lau and Kan, 1983; El-Gewely and Oxender, 1985]. The subclone of the G418-resistant CHO–*ts*025C1 cell line used in this study is referred to as CHO–R100 [El-Gewely and Oxender, 1985]. The regulatory mutant cell line, CHO–C11B6, is both $HPRT^+$ and G418 sensitive, which permitted us to select hybrids between the CHO–R100 and CHO–C11B6 cell lines. Hybrids were selected in medium containing hypoxanthine, aminopterin, thymidine (HAT supplement), and G418. The frequency of successful fusion was approximately 1 in 10^3 cells. Control fusions of either CHO–R100 × CHO–R100 cells and CHO–C11B6 × CHO–C11B6 cells did not result in any surviving colonies in selective medium.

To be certain that the surviving colonies were hybrids, the number of chromosomes in the parental cells and the putative hybrid cells were determined. The average number of chromosomes in both the CHO–R100 and CHO–C11B6 cell lines was 20. Four cell lines that were produced by the selection, designated R × C 1.5, 1.6, 2.1, and 2.5, had average chromosome numbers of 40, 39, 35, and 39, respectively. The near-tetraploid chromosome number implied that these cell lines were hybrids. The hybrid cell lines were maintained in selective medium at 34°C for further analysis.

Regulation of Leucine Transport Activity in the Hybrid Cell Lines

Figure 1 shows the initial rate of Na^+-independent leucine uptake by the CHO–R100, CHO–C11B6, and hybrid cell lines, after a 9-h incubation at either 34°C or 39°C. The CHO–R100 cell line showed temperature-dependent regulation of leucine uptake, while the CHO–C11B6 cell line showed elevated and unregulated transport activity. The hybrid cell lines all demonstrated temperature-dependent regulation of leucine uptake. For the hybrid cell lines, as well as for the CHO–R100 cell line, the rate of leucine uptake after incubation at 39°C was two- to threefold higher than that seen after incubation at 34°C. The initial rates of leucine uptake in the hybrid cell lines were greatly reduced compared to the CHO–C11B6 cell line, although they were

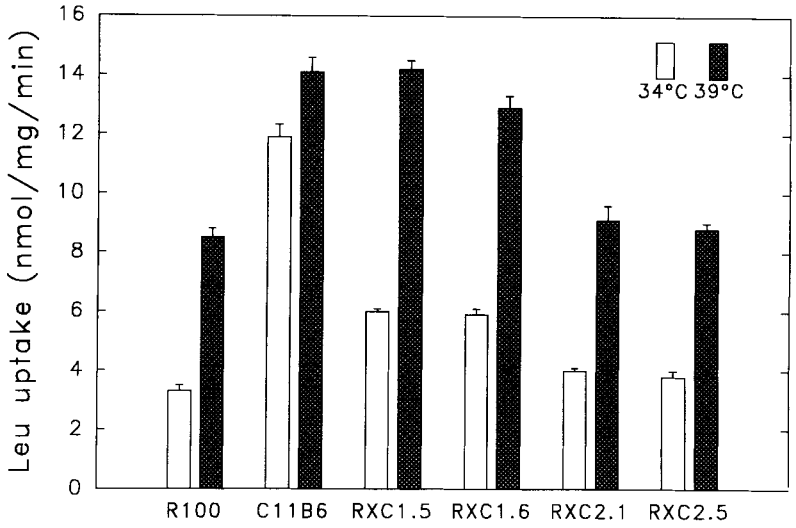

Fig. 1. Regulation of Na^+-independent leucine uptake in the CHO–R100, CHO–C11B6, and CHO–R100 × CHO–C11B6 hybrid cell lines. Cells were plated at either 1.4×10^5 cells/well (the CHO–R100 and CHO–C11B6 cell lines), or at 7×10^4 cells/well (the hybrid cell lines) and grown in MEM at 34°C. One-half the dishes were then moved to 39°C and one-half left at 34°C. After 9 h, the 30-s Na^+-independent uptake of 50 μM leucine was determined. Data are the averages of four determinations.

slightly higher than that seen for the CHO–R100 cell line.

Time Course of Leucine Uptake in the Hybrid Cell Lines

The time course of the Na^+-independent uptake of 50 μM leucine was measured in the CHO–R100, CHO–C11B6, and hybrid cell lines at 34°C (Fig. 2). The steady-state accumulation of leucine over 5 min by the hybrid cell lines was similar to that seen in the CHO–R100 cell line, indicating that the lower initial rate of leucine uptake at 34°C results in a lower steady-rate accumulation of leucine by the hybrid cell lines.

Kinetics of Leucine Uptake by the CHO–R100, CHO–C11B6, and Hybrid Cell Lines

We examined the kinetic parameters of Na^+-independent leucine uptake by the parental cell lines and the hybrid cell lines (Table I). The increased leucine uptake seen by the CHO–R100 cell line at 39°C was accompanied by a twofold increase in the V_{max} value after incubation at 39°C over that seen after incubation at 34°C. The CHO–C11B6 cell line showed elevated V_{max} values at both temperatures, compared to CHO–R100, as reported previously [Shotwell et al., 1983]. Because the K_m values for the CHO–C11B6 cell line are also slightly higher that those for the CHO–R100 cell line, the data are also expressed as the ratio V_{max}/K_m,

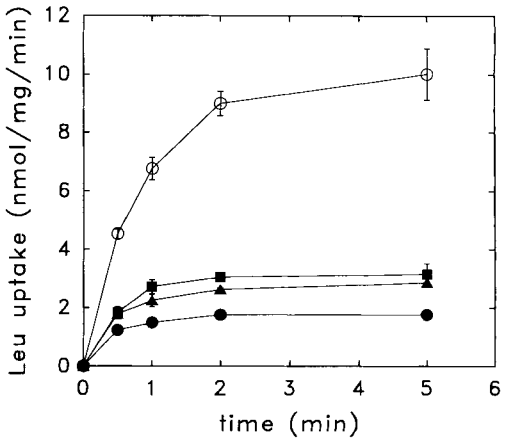

Fig. 2. Time course of Na^+-independent leucine uptake at 34°C in the CHO–R100, CHO–C11B6, and CHO–R100 × CHO–C11B6 hybrid cell lines. Cells were plated at either 1.4×10^5 cells/well (the CHO–R100 and CHO–C11B6 cell lines) or 7×10^4 cells/well (the hybrid cell lines) and grown in MEM at 34°C. The Na^+-independent uptake of 50 μM leucine was determined at the indicated times. Points are the averages of four determinations: ○, CHO–C11B6; ●, CHO–R100; ■, R × C 1.5; ▲, R × C 2.5.

which is proportional to the actual rate of uptake at low substrate concentrations. These values show that the CHO–C11B6 cell line has a greatly increased rate of uptake over that seen for the CHO–R100 cell line. The hybrid cell lines R × C 1.5 and R × C 2.5 showed an increase in V_{max} values after incubation at 39°C over those seen after incubation at 34°C, similar to the properties of the CHO–R100 cell line, although

TABLE I. Kinetics of Na^+-Independent Leucine Uptake by the CHO–R100, CHO–C11B6, and Hybrid Cell Lines*

Cell line	Temp. °C	K_m (mM)	V_{max} (nmol/mg/min)	P (nmol/mg/min/mM)	V_{max}/K_m (nmol/mg/min/mM)
CHO–R100	34	0.016	3.2	4.8	200
	39	0.023	6.3	6.3	274
CHO–C11B6	34	0.038	14.7	5.5	387
	39	0.058	18.9	4.0	326
R × C 1.5	34	0.021	6.1	7.1	290
	39	0.032	14.7	7.3	459
R × C 2.5	34	0.019	4.7	4.8	247
	39	0.023	8.0	5.2	348

*Cells were depleted of internal amino acids at 34°C. They were then plated and grown in MEM at 34°C. After 8 h at 34°C or 39°C, the cells were depleted of internal amino acids, and the 30-s uptake of leucine at concentrations of 5 μM–2 mM was determined in sodium-free buffer.

the hybrid cell lines had higher V_{max} values than those of the CHO–R100 cell line at 34°C. The V_{max}/K_m values for the hybrid cell lines also show that these cell lines have a regulated phenotype similar to that of the CHO–R100 cell line.

Plating Efficiency of the CHO–R100, CHO–C11B6, and Hybrid Cell Lines

We examined whether the hybrid cell lines also demonstrated temperature-sensitive growth. The temperature sensitivity of the two hybrid cell lines was tested by comparing their plating efficiencies to those of the CHO–R100 and CHO–C11B6 cell lines at 34°C and 39°C (Table II). CHO–R100 cells could only be plated successfully at 34°C. CHO–C11B6 cells plated equally well at both temperatures, in agreement with previous results [Shotwell et al., 1983]. The hybrid cells only plated well at 34°C, indicating that they were temperature sensitive.

TABLE II. Plating Efficiency of the CHO–R100, CHO–C11B6, and CHO–R100 × CHO–C11B6 Hybrid Cell Lines*

Cell line	34°C	39°C
CHO–R100	87	0
CHO–C11B6	114	124
R × C 1.5	69	0
R × C 2.5	78	4

*Plating efficiency was determined as described under Materials and Methods. Numbers are the averages of two plates.

DISCUSSION

We have been using genetic approaches, as well as kinetic analyses, to examine amino acid transport system L activity and its regulation. The temperature-sensitive leucyl-tRNA synthetase mutant cells CHO–*ts*H1 and CHO–*ts*025C1 can be starved for leucine at elevated temperatures, and consequently increase their system L activity [Moore et al., 1977; Shotwell et al., 1982; Lobaton et al., 1984]. Mutants isolated from the CHO–*ts*H1 cell line show increased and unregulated system L transport activity, suggesting that these cell lines are defective in a regulatory element that leads to constitutively elevated system L transport activity [Shotwell et al., 1983]. Fusion of the regulatory mutant cells with cells able to regulate system L produced hybrid cells that were also able to regulate system L transport activity in response to amino acid restriction. The system L transport properties of the hybrid cell lines were similar to the regulated transport phenotype characteristic of the CHO–R100 cell line. These results suggest that CHO–R100 cells contain a dominant element that can regulate the system L transport activity contributed by the two parental cell lines. The slightly higher level of leucine transport activity shown by the hybrid cell lines over that seen for CHO–R100 (e.g., Fig. 1) may result from a limitation in the amount of the regulatory element such that the hybrid cells cannot completely repress their transport activity.

Previously, we provided evidence suggesting that the enhancement of system L activity in the CHO–*ts*H1 cell line at elevated temperatures is regulated at the level of translation [Shotwell et al., 1982]. The constitutively elevated system L transport activity seen in the CHO–C11B6 cell line could be the result of the loss of a regulatory element that acts as a repressor at the level of translation, possibly responding to the level of charged leucyl-tRNA. Alternatively, the regulatory factor may modify transport activity at the membrane. The latter possibility is interesting in light of the recent cloning of rabbit and rat cDNAs which, when injected into *Xenopus* oocytes, result in enhanced neutral and cationic amino acid uptake into the oocytes [Tate et al., 1992; Wells and Hediger, 1992; Bertran et al., 1992]. The authors concluded that the cloned component may be a regulatory element or a component of a transport complex that can activate an endogenous but inactive oocyte transport system, rather than serving as an actual transporter molecule itself, since the deduced

amino acid sequence of the rabbit kidney cDNA suggests there is only one membrane-spanning domain [Wells and Hediger, 1992; Bertran et al., 1992]. CHO cell mRNA was recently used in an oocyte expression system and resulted in enhanced Na^+-independent leucine uptake as well as increased uptake of other neutral and cationic amino acids [Su et al., 1992]. The eventual cloning of leucine transport components should help elucidate the molecular basis of system L transport properties.

Although temperature-sensitive synthetase mutant cell lines show increased leucine transport activity at the nonpermissive temperature, the increase in transport activity still does not allow these cells to survive at elevated temperatures, possibly because of the delay (6–8 h) in reaching maximum transport activity and increased intracellular leucine levels. The regulatory mutant cell lines may be temperature resistant because they are able to maintain high intracellular leucine levels. The hybrid cell lines, like the CHO–R100 cell line, are able to regulate leucine transport activity, which results in a temperature-sensitive phenotype. These results support the hypothesis that the temperature sensitivity or resistance of cells with a defective leucyl-tRNA synthetase is determined by the level of system L transport activity of the cells.

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