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Genetics

Tritium suicide selection of mammalian cell mutants defective in the transport of neutral amino acids

(cell membrane/α-aminoisobutyric acid/lymphocytes)

MORRIS C. FINKELSTEIN*, CAROLYN W. SLAYMAN, AND EDWARD A. ADELBERG

Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **Mouse lymphocytic cells of the established line GF-14 were allowed to accumulate intracellular ^{3}H-labeled aminoisobutyric acid (AIB), frozen, and stored over liquid N_2. After internal radiation had reduced survival to 1 in 10^4, survivors were plated and tested for their ability to transport AIB. Out of 200 clones tested, two (designated GF-17 and GF-18) were found to have reductions to 13–35% of the parent in the rate of transport of AIB, L-alanine, L-proline, and L-serine; GF-18 also showed significant reductions in the rate of transport of L-glutamate and DL-cysteine. Little or no change was observed for 10 other amino acids or for thymidine. Kinetic analyses revealed that the mutants were not altered in K_m for AIB uptake, but had V_{max} values approximately 20% the value of the parent strain, GF-14, suggesting that either the number of AIB transport sites or the turnover rate of the sites has been reduced in the two mutants.**

Because mammalian cells possess multiple amino acid transport systems with overlapping substrate specificities (1), it has proven difficult to characterize any single system with respect to its kinetics, physiological function, or molecular components. Such characterizations have been facilitated in microorganisms by the isolation of transport mutants (2); comparatively little progress has been made, however, toward the genetic study of amino acid transport in mammalian cells. Although a variety of human inborn disorders of amino acid transport have been described [including cystinuria, Hartnup disease, Lowe syndrome, and iminoglycinuria (for review, see ref. 3)], these disorders appear to involve specialized transport processes expressed only in the kidney and/or the small intestine, and do not affect amino acid transport in cells, such as lymphocytes or fibroblasts, that can be more easily studied. Recently, however, several amino acid transport mutants have been isolated in mammalian fibroblastic cell lines by selecting for resistance to amino acid analogues (4) or to high concentrations of a naturally occurring amino acid (5).

In this paper, we describe a new approach to the selection of mammalian cell mutants defective in amino acid transport. We have successfully isolated two such mutants of mouse lymphocytes by means of tritium suicide, patterned after the ^{32}P suicide procedure used by Harold *et al.* to isolate PO_4 transport mutants of *Streptococcus faecalis* (6). In that procedure, cells were treated with a mutagen, exposed to a radioactive substrate at extremely high specific activity, frozen, and stored. Normal cells (having accumulated a high intracellular concentration of substrate) were killed by radioactive decay, while transport mutants (having failed to accumulate substrate) survived selectively. This method was expected to yield amino acid transport mutants of cultured mammalian cells, because a modified version of tritium suicide had been used to select thymidine transport mutants of Chinese hamster cells (7), and the accumulation of ^{3}H-labeled amino acids had been shown to kill mouse lymphocytic cells of strain L5178Y during storage at −196° (8).

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The mouse lymphocytic cell line GF-14 was used for the isolation of transport mutants. GF-14 was derived from strain L5178Y (9) by three sequential induced mutations, making it asparagine synthetase-positive, thymidine kinase-deficient, and resistant to 1 mM ouabain.

Cells were grown at 37° in spinner bottles containing RPMI 1640 medium adapted for equilibration with air by the following modifications: $NaHCO_3$ (4.17 mM), oxalacetic acid (1.0 mM), sodium pyruvate (0.45 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes; 25 mM), uridine (0.04 mM), and hypoxanthine (0.1 mM). Horse serum was added to a final concentration of 2.5% (vol/vol).

Mutagenesis. Logarithmic-phase cells (2.5×10^5/ml) were treated with ethyl methanesulfonate (300 μg/ml) in growth medium for 20 hr. The cells were washed twice with Earle's balanced salt solution and resuspended at 4×10^4 cells per ml in fresh growth medium containing 15% horse serum. They were then passaged for 4 days in spinner culture, during which time they underwent approximately six doublings.

Amino Acid Uptake. The initial rates of uptake of radioactively labeled amino acids were measured by the "silicone sandwich" technique (10), following the procedure described in our previous paper (11). In this procedure, the cells are separated from the incubation medium by rapid centrifugation through a silicone layer into 12% perchloric acid; the amount of radioactive amino acid accumulated by the cells is then measured by analyzing samples of the perchloric acid cell extract in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

α-Aminoisobutyric acid (AIB), a nonmetabolizable amino acid, is actively transported by GF-14 cells (11). In order to select for mutants deficient in AIB transport, GF-14 cells were mutagenized, regrown as described above, and incubated at 4×10^6 cells per ml for 50 min at 37° in Earle's balanced salt solution containing 8.0 μM [*methyl*-^{3}H]AIB at 2.5 Ci/mmol.

By the end of the incubation, the intracellular AIB concentration had reached 0.167 pmol/10^6 cells, sufficient to produce 1330 decays/cell per day. The cells were then frozen by a modification of the method of Farrant *et al.* (12) in the presence of 10% glycerol (vol/vol), and stored at −100° over liquid N_2.

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Abbreviations: AIB, α-aminoisobutyric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Present address: Department of Medicine, New York University Medical Center, New York, NY 10016.

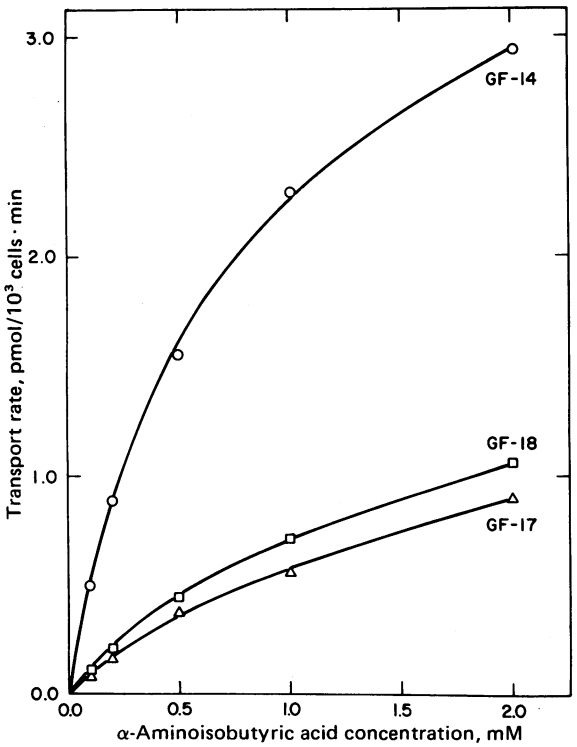

FIG. 1. Concentration dependence of AIB uptake in the parental strain, GF-14, and in the mutants, GF-17 and GF-18. Logarithmic-phase cells were harvested, washed, resuspended in ice-cold incubation medium, distributed into glass tubes, and maintained at 0° in an ice bath for up to 1 hour. The incubation medium was Earle's balanced salt solution with the following modifications: 25 mM Hepes, 4.17 mM $NaHCO_3$, and 140 mM NaCl. The incubation medium had a pH of 7.4 and an osmolality of 300 mOsm. For each transport measurement, one tube was moved to a reciprocating shaking water bath at 37° and preincubated for 5 min. At zero time, an aliquot of the cell suspension was withdrawn and added to a vial of incubation medium containing [*methyl*-^{3}H]AIB at 8.0 μCi/ml plus unlabeled AIB to give the desired final concentration, and maintained in the shaking water bath. At 20-sec intervals, 200-μl aliquots were withdrawn, and the cells were separated from the incubation medium by centrifugation through silicone into perchloric acid. The tubes were cut immediately below the silicone layer, and samples of the aqueous and perchloric acid layers were removed and analyzed in a liquid scintillation spectrometer. The initial rates of uptake were determined by least squares fit of the data; each rate plotted is the mean of four determinations, each consisting of four points spaced at 20-sec intervals.

Aliquots were thawed periodically and cloned in RPMI 1640 medium containing 15% horse serum and 0.12% agar. After 4 months, by which time survival had decreased by a factor of 10^4, approximately 200 surviving clones were isolated and tested for their ability to transport 0.20 mM [*methyl*-^{3}H]AIB. Of the clones tested, two (designated GF-17 and GF-18) showed a significant reduction in AIB uptake and were saved for further study.

In order to determine the scope of the transport defect in GF-17 and GF-18, a preliminary survey was made of the uptake of 16 radioactive amino acids and thymidine in these two strains and in the parental strain GF-14; the radioactive substrates were used at concentrations ranging from 0.2 to 20 μM. The survey revealed a striking and specific defect in the transport of small, neutral amino acids: the initial rate of uptake of AIB, L-alanine, L-serine, and L-proline was reduced to 13–30% in GF-17 and to 16–35% in GF-18. The uptake of DL-cysteine and L-glutamic acid was reduced in GF-18 (to 56% and 55%, respectively) but was not reduced in GF-17,

Table 1. Parameters of α-aminoisobutyrate uptake

Cell line	V_{max} ± SEM, pmol/10^3 cells·min	K_m ± SEM, mM	K_d ± SEM,* pmol·liter/mmol·10^3 cells·min
GF-14	3.14 ± 0.11	0.57 ± 0.03	0.25 ± 0.02
GF-17	0.50 ± 0.06		
GF-18	0.70 ± 0.06		

* The diffusion constant, K_d, may be simplified so as to equal 0.395 min^{-1}, based on an estimated intracellular volume for GF-14 and its derivatives GF-17 and GF-18 of 6.43×10^{-13} liter per cell (11).

suggesting that the two strains are genetically different. The uptake of the remaining compounds tested (L-leucine, L-methionine, L-threonine, L-arginine, L-lysine, L-phenylalanine, L-tyrosine, L-isoleucine, L-aspartic acid, glycine, and thymidine) was reduced to a much lesser extent or not at all, in either strain.

These results point to a specific defect in a transport system for which the major substrates are small, neutral α-amino acids. Such a system has been identified by kinetic inhibition studies in Ehrlich ascites cells (1), and in cultured mouse lymphocytes (11). In both cases, glutamic acid has been identified as an additional substrate (13, 14).

In order to obtain quantitative information about the transport defect, AIB uptake was measured over a wide range of extracellular concentrations (from 0.1 to 2.0 mM) in the parental strain GF-14 and in the two mutants, GF-17 and GF-18. The results are illustrated in Fig. 1. The curves were drawn on the basis of a computer fit of the data (15) to the sum of a Michaelis–Menten component and a linear component (presumably diffusion):

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

in which v is the initial rate of uptake, S is the extracellular AIB concentration, V_{max} and K_m are the kinetic constants of the Michaelis–Menten component, and K_d is a rate constant for the linear uptake component.

In preliminary computer trials, no significant differences were obtained for K_m or K_d among the three strains; a joint fit of the data was then run holding K_m and K_d constant and allowing V_{max} to vary. The results (Table 1) show that V_{max} for AIB transport is reduced to 16% in GF-17 and to 22% in GF-18, compared with the parental cell line, GF-14. One can infer that either the number of AIB transport sites or the turnover rate of the sites has been reduced in the two mutants.

This defect may explain the lower growth rates of the mutants: the generation times for GF-17 and GF-18 in a modified RPMI 1640 medium (described in *Materials and Methods*) were 19.2 hr and 21.3 hr, respectively, in comparison with a generation time of 14.4 hr for the parental strain, GF-14. The mutant phenotypes have remained stable over several months of serial culture.

The kinetics of residual transport of a number of affected amino acids in GF-17 and GF-18 can now be analyzed, in order to determine the nature and scope of the genetic defect more clearly, and to reveal those overlapping transport systems that have been unmasked by the reduced activity of the major neutral amino acid transport system. Further mutant screenings based on tritium suicide can be carried out, using the behavior of the mutants in reconstruction experiments as a guide to more efficient selection conditions.

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