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Effects of Ecotropic Murine Retroviruses on the Dual-function Cell Surface Receptor/Basic Amino Acid Transporter*

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The widely expressed Na⁺-independent transporter for basic amino acids (system y⁺) is the cell surface receptor (ecoR) for ecotropic host-range mouse retroviruses (murine leukemia viruses (MuLVs)), a class of retroviruses that naturally infects only mice or rats. Accordingly, expression of mouse ecoR cDNA in mink CCL64 fibroblasts yields cells (CEN cells) that have y⁺ transporter activity above the endogenous background and that bind and are infected by ecotropic MuLVs. The effect of ecotropic MuLV infection on expression of y⁺ transporter was analyzed in mouse and in mink CEN fibroblasts. Chronic infection with ecotropic MuLVs caused 50–70% loss (down-modulation) of mouse y⁺ transporter in plasma membranes, detected as a reduced V_{max} for uptake and outflow of L-[³H]-arginine with no effect on K_m values. Down-modulation was specific for mouse y⁺ and did not affect other transporters or the endogenous mink y⁺, suggesting that it results from specific interaction between mouse y⁺ and the viral envelope glycoprotein gp70 in the infected cells. Because this partial loss of mouse y⁺ from cell surfaces is insufficient to explain the complete interference to superinfection that occurs in cells chronically infected with ecotropic MuLVs, alternative explanations for interference are proposed. In contrast to the y⁺ down-modulation caused by chronic infection, binding of extracellular envelope glycoprotein gp70 at 37 °C resulted in noncompetitive inhibition of amino acid import by mouse y⁺ but had no effect on export through this same transporter or on any transporter properties of mink y⁺. The effects of gp70 on transport kinetics suggest that it slows the rate-limiting step of the amino acid import cycle, a conformational transition of the empty transporter in which the binding site moves from the inside back to the outside of the cell, and that gp70 has no effect on the rate-limiting step of the amino acid export cycle. Infected cells retain substantial y⁺ activity. Moreover, the virus binding site on ecoR is in a mobile region that changes conformation during the amino acid transport cycle.

porter for basic amino acids lysine, arginine, and ornithine (system y⁺) (1, 2). These studies used *Xenopus laevis* oocytes that were injected with messenger RNA transcribed from cloned ecoR cDNA (3). The transport activities, detected using radioactive amino acid uptake (1, 2) and electrophysiological measurements (2), corresponded with previously characterized properties of system y⁺ (4–7).

These results raise important questions pertaining to interactions between the dual functions of this “Trojan Horse” (8) ecoR/y⁺ transporter. For example, interference enables cells that synthesize a viral envelope glycoprotein to efficiently resist superinfection by any retrovirus that uses the same cell surface receptor (9). For several viruses, including human immunodeficiency virus (HIV) (10–13) and avian reticuloendotheliosis virus (14), interference involves depletion of receptors from surfaces of infected cells. Apparently, complexes of the receptor and the envelope glycoprotein form in the rough endoplasmic reticulum shortly after protein synthesis, and these complexes are degraded intracellularly. In the case of HIV, a virus-encoded protein Vpu stimulates degradation of the cell surface receptor CD4 (15). However, elimination of y⁺ transporter from plasma membranes would probably be lethal to cells that lack alternative transporters for essential basic amino acids. Conceivably, starvation for amino acids could be involved in aspects of pathogenesis that have been mapped to the envelope gene of ecotropic MuLVs, such as hemolytic anemia, immunodeficiency, and neural degeneration (16–19).

Although we previously found that adsorption of the gp70 envelope glycoprotein from an ecotropic MuLV had no effect on ecoR/y⁺ transporter activity in *Xenopus* oocytes at 25 °C (2), we emphasized that this result was inconclusive because gp70 binding to ecoR is highly temperature-dependent (20, 21). As is the case with the envelope glycoprotein encoded by HIV (22), binding at 37 °C could be qualitatively as well as quantitatively different from that at 25 °C.

We have addressed these issues by comparing y⁺ transporter functions in uninfected and infected mammalian cells and in uninfected cells incubated with ecotropic gp70 at 37 °C. We observed several inhibitory effects of ecotropic MuLV and of its *env* glycoprotein gp70 that are specific for the mouse y⁺ transporter.

MATERIALS AND METHODS

Cells and Viruses—Mouse embryo Balb/c 3T3 fibroblasts and mink lung CCL64 fibroblasts were from the American Type Culture Collection. ψ 2 ecotropic packaging cells line (23) and PA12 amphotropic packaging cells (24) were from R. C. Mulligan (MIT, Cambridge, MA) and A. D. Miller (Fred Hutchinson Cancer Center, Seattle, WA), respectively. CEN cells, a derivative of CCL64 cells that express ecoR on their surfaces, have been described previously (25). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Recent studies suggested that the cell surface receptor (ecoR) for ecotropic host-range murine leukemia viruses (MuLVs)¹ is the widely expressed Na⁺-independent trans-

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¹ The abbreviations used are: MuLV, murine leukemia virus; HIV, human immunodeficiency virus; EBSS, Earle's balanced salt solution; PBS, phosphate-buffered saline; hGH, human growth hormone.

Ecotropic Rauscher murine leukemia virus (R-MuLV) (26) and dualtropic MCF-13 virus (27) were described previously. R-MuLV pseudotyped with an amphotropic host-range envelope was obtained from PA12 cells that were infected with R-MuLV. Helper-free ecotropic host-range virus that encodes human growth hormone was described elsewhere (25, 28). Virus preparations were obtained by removing medium that had been placed on half-confluent monolayers of virus-producing cell lines 16 h earlier and by filtering it through a 0.2- μm pore filter. Viral infections were done by incubating 10^6 cells in 25-cm² flasks with 1 ml of virus-containing medium for 2 h at 37 °C in the presence of Polybrene (8 $\mu\text{g}/\text{ml}$).

Amino Acid Transport Assays—The amino acid transport assay was based on the method initially described by Gazzola *et al.* (29). All washes of the monolayers and measurements of amino acid uptake were done in Earle's balanced salt solution (EBSS) (1.8 mM CaCl₂, 5.3 mM KCl, 0.8 mM MgSO₄, 117 mM NaCl, 1 mM NaH₂PO₄, 5.6 mM D-glucose, 0.03 mM phenol red, pH 7.4) except in the experiments where choline chloride and choline hydrogen phosphate replaced sodium chloride and sodium hydrogen phosphate, respectively. Choline hydrogen phosphate was prepared by stoichiometric titration of 250 mM choline bicarbonate with phosphoric acid followed by boiling to remove CO₂.

L-[2,3,4,5-³H]Arginine and L-[2,3,4,5-³H]proline were from Amersham. 10^6 cells were plated in 2-cm² wells of a 24-well cluster dish 2 days before transport assay. Cells were washed with amino acid-free EBSS and then exposed to the radioactive substrate for 0.5 to 1 min at 37 °C. L-[³H]Arginine in EBSS with a specific activity of 50 $\mu\text{Ci}/\mu\text{mol}$ was used in uptake experiments. After incubation with radioactive solute, cells were washed rapidly twice with 2 ml of ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄) and extracted with 200 μl of 5% trichloroacetic acid, and the soluble phase was then counted in a liquid scintillation counter. The protein concentration of each culture was measured by the Bio-Rad protein assay (Bio-Rad) after dissolving the 5% trichloroacetic acid-insoluble phase of the culture in 200 μl of 0.1 N NaOH. The rates of amino acid uptake for individual cultures were standardized to their protein concentrations and fitted by least squares to the Michaelis-Menten equation. The least-squares fitting analysis resulted in computer-derived K_m and V_{max} estimates \pm S. E. of the estimate. These values are indicated in the figure legends.

For measuring initial rate of outflow of an amino acid, cells plated in the 24-well dish as described above were washed once with 2 ml of amino acid-free EBSS and then incubated with 200 μl of 1 mM L-[³H]arginine (50 $\mu\text{Ci}/\mu\text{mol}$) for 1.5 h at 37 °C. Cells were rapidly washed three times with 2 ml of ice-cold EBSS before 1 ml of EBSS was added to the cells at 37 °C. Samples taken at different times were measured for radioactivity in a liquid scintillation spectrometer. Radioactivity measured in efflux was standardized to the protein concentration of individual cultures as described above.

Adsorption of gp70 that had been purified from ecotropic Friend MuLV was done at a concentration of 4 $\mu\text{g}/\text{ml}$ in culture medium at 37 °C for 2 h, as described previously (20, 21, 25).

Assay for gp70 Expression on the Surfaces of Infected Cells—Cells (10^6) were plated onto coverslips in 6-well (9.5 cm²) dishes 1 day before analysis. Cells were sequentially incubated for 1 h at 37 °C with a 1:200 dilution of goat antiserum made to gp70 (30) and then for 1 h with a 1:200 dilution of fluorescein-conjugated rabbit anti-goat antibody (Zymed Laboratories, Burlingame, CA). After cells were rinsed three times with culture media and once with PBS, they were fixed with cold methanol, rinsed with PBS, and mounted for fluorescence microscopy.

Quantitative Analysis of Ecotropic Virus Infection—Quantitative analysis of ecotropic virus infections by using the helper-free virus that encodes human growth hormone (hGH virus) has been described previously (25). Briefly, cells infected 48 h earlier with the helper-free hGH virus were plated on coverslips in 9.5-cm² dishes 1 day before analysis. Cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. Dilutions of rabbit antiserum to hGH (donated by the National Institute of Diabetes and Digestive and Kidney Diseases) (1:3,000) and of fluorescein-conjugated goat anti-rabbit immunoglobulin (Tago Immunologicals, Burlingame, CA) (1:200) were sequentially incubated with the cells for 1 h at 37 °C. After washing with media three times and PBS once, the cells were mounted for immunofluorescence microscopy. The fraction of fluorescent cells was determined by analyzing at least 10 to 15 microscopic fields that contained a total of at least 200 fluorescent cells. Multiplicity of infection was calculated from the binomial

distribution as $-\log P/0.44$, where P is the fraction of nonfluorescent cells (25, 28).

RESULTS

***y*⁺ Transporter Activities in Uninfected and Infected Mouse Fibroblasts**—Initial studies strongly suggested that mouse Balb/c 3T3 fibroblasts contain an amino acid transporter with properties consistent with system *y*⁺ plus a nonsaturable component for L-[³H]arginine uptake. For example, as illustrated in Fig. 1A, the uptake had a high affinity component with a K_m of approximately 60–100 μM as described for *y*⁺ (1, 2, 4, 5), and an additional nonsaturable component that imparted a secondary slope to the L-[³H]arginine saturation curve. Similar secondary slopes have been reported for some other cells (4–7). Furthermore, as described in Fig. 2, uptake of 200 μM L-[³H]arginine was Na⁺-independent and was inhibited by L-lysine but not by L-proline. A unique characteristic of system *y*⁺ is that it transports homoserine and several other neutral amino acids with a low affinity (*i.e.* $K_m \sim 25$ mM) by a Na⁺-dependent co-transport mechanism (2, 4–7). Uptake of L-[³H]arginine into Balb/c 3T3 fibroblasts was partially inhibited by 10 mM homoserine in the presence of Na⁺ (Fig. 2A), but not in its absence (Fig. 2B).

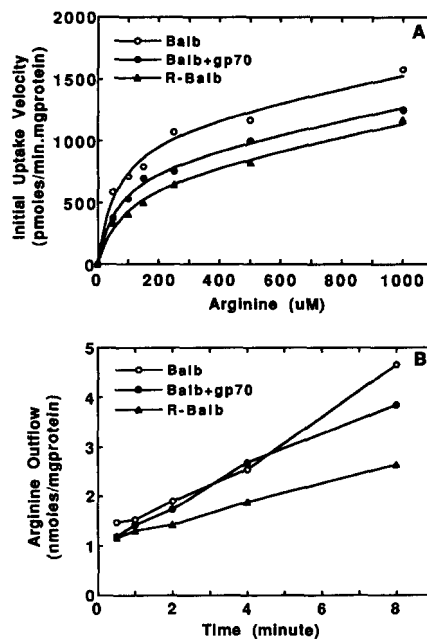


FIG. 1. Measurements of L-[³H]arginine uptake and outflow in mouse Balb/c 3T3 fibroblasts. Uptake and outflow of L-[³H]arginine were measured in uninfected mouse Balb/c 3T3 (Balb, ○), Balb/c 3T3 infected with ecotropic Rauscher MuLV (R-Balb, ▲), and uninfected Balb/c 3T3 that contain adsorbed ecotropic gp70 (Balb+gp70, ●). **Panel A** shows uptake of L-[³H]arginine. The cells were washed thoroughly and then exposed to different concentrations of L-[³H]arginine (50 $\mu\text{Ci}/\mu\text{mol}$) at 37 °C for 1 min. After rapidly washing twice with 2 ml of ice-cold PBS, the radioactive arginine accumulated in the cells was extracted with 5% trichloroacetic acid as described under "Materials and Methods." The lines in panel A were drawn by computer using least-squares fitting to the equation $v = V_{\text{max}}[S]/(K_m + [S]) + 0.48[S]$. The computer-derived K_m estimates were 61 ± 13 μM , 66 ± 8 μM , and 88 ± 15 μM for the uninfected cells, uninfected cells with adsorbed gp70, and infected cells, respectively. The corresponding V_{max} values for these same cells were 1110 ± 58 , 836 ± 27 , and 713 ± 33 pmol/min/mg of protein, respectively. **Panel B** shows outflow of L-[³H]arginine from cells preloaded by incubation for 1.5 h at 37 °C with a 1 mM concentration of the radioactive amino acid. For such highly preloaded cells, the initial rate of outflow should be constant (as observed) and equal to V_{max} for export. Both uptake and outflow experiments were repeated once, with similar results.

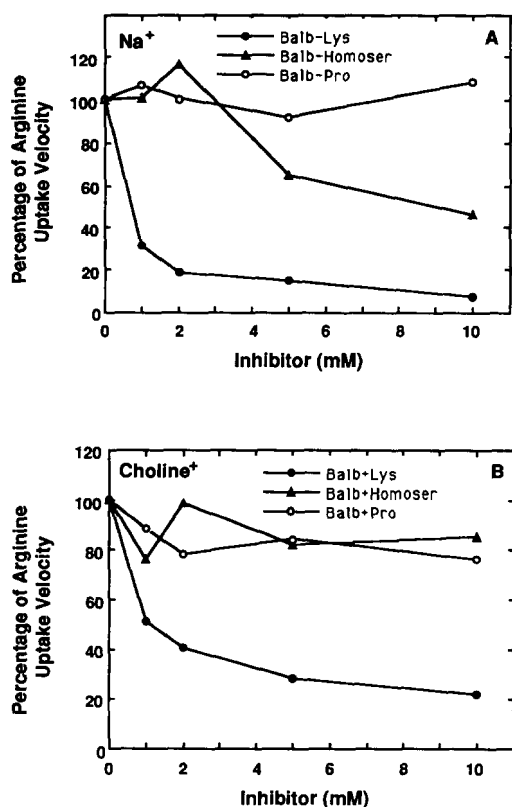


FIG. 2. Inhibition of L-[³H]arginine uptake into Balb/c 3T3 fibroblasts by lysine, homoserine, and proline in the presence (panel A) or absence (panel B) of sodium. Cells were washed and then exposed to 200 μ M L-[³H]arginine (50 μ Ci/ μ mol) in the presence of inhibitors at different concentrations for 1 min at 37 °C. In panel B, the EBSS medium contained choline chloride and choline hydrogen phosphate instead of sodium chloride and sodium hydrogen phosphate. The data are plotted as the percentage of the uptake observed in the absence of inhibitors. The 100% values for L-[³H]arginine uptake were 710 and 855 pmol/min/mg of protein from panels A and B, respectively.

As shown in Fig. 1A, chronic infection of the Balb/c 3T3 fibroblasts with ecotropic MuLV (greater than 99% of the cells were infected as determined by immunofluorescence for viral gp70) or saturation binding of the ecotropic envelope glycoprotein gp70 to the cells at 37 °C caused substantial but incomplete inhibitions of the y^+ component of L-[³H]arginine uptake. These inhibitions were noncompetitive since they were not overcome by high concentrations of L-[³H]arginine. The initial rate of efflux of L-[³H]arginine from preloaded cells was also reduced substantially in infected compared with uninfected cells (Fig. 1B). However, gp70 adsorption onto the cells had no significant effect on efflux of the amino acid.

Although significant and reproducible, these effects of virus and of gp70 were difficult to quantitatively evaluate. In order to perform Michaelis-Menten analysis, it was necessary to subtract the nonsaturable component of uptake. For the results in Fig. 1A, such subtraction suggests that infection with ecotropic MuLV reduces y^+ activity by approximately 50%, and that adsorption of extracellular gp70 reduces y^+ import activity by approximately 25%. It is also difficult with these cells to be certain that all of the high affinity L-[³H]arginine uptake is due to system y^+ ; some cells contain another high affinity Na⁺-independent transporter (31). Consequently, these studies of Balb/c 3T3 fibroblasts do not establish whether infection causes complete loss of y^+ with residual activity due to another transporter or whether it causes only partial depletion of y^+ .

y^+ Transporter Activities in Mink CCL64 Fibroblasts—To circumvent these problems inherent in studies using mouse cells, we analyzed y^+ transport using CCL64 mink lung fibroblasts and a derivative cell line (CEN) that expresses a large quantity of recombinant mouse *ecoR* (25). Fig. 3 shows an initial comparison of L-[³H]arginine uptake into CCL64 cells, CEN cells, CEN cells chronically and efficiently (greater than 99%) infected with the Rauscher strain of ecotropic MuLV (R-CEN), and CEN cells that had adsorbed extracellular gp70 at 37 °C. These data are plotted as $1/v$ versus $1/[S]$ in Fig. 3, inset. Clearly, CEN cells that express *ecoR* contain substantially more apparent y^+ transporter activity than the CCL64 cells that contain only the endogenous mink transporter. Although the chronically infected R-CEN cells have much less y^+ than the uninfected CEN cells, they nevertheless contain more than the background amount that occurs in the control CCL64 cells. This implies that infection causes incomplete loss of mouse y^+ . Moreover, as with mouse fibroblasts (Fig. 1), adsorption of gp70 at 37 °C onto CEN cells causes partial (approximately 25%) noncompetitive inhibition of L-[³H]arginine uptake. Support for these conclusions is described below.

This mink cell system is advantageous for analyzing ecotropic MuLV effects on y^+ transporter activities. First, because CEN cells express recombinant mouse *ecoR*, there is no doubt that the difference between CEN and CCL64 cells is due to mouse y^+ . Second, the L-[³H]arginine uptake curve for CCL64 mink cells has a simple shape consistent with the Michaelis-Menten equation, and it lacks the secondary slope that occurs in Balb/c 3T3 cells. Indeed, the endogenous transport activity in mink CCL64 cells has properties consistent with system y^+ , including its K_m for arginine, Na⁺ independence, inhibition by other basic amino acids, and Na⁺-dependent inhibition by a high concentration of homoserine (results not shown). Third, as shown below, the endogenous

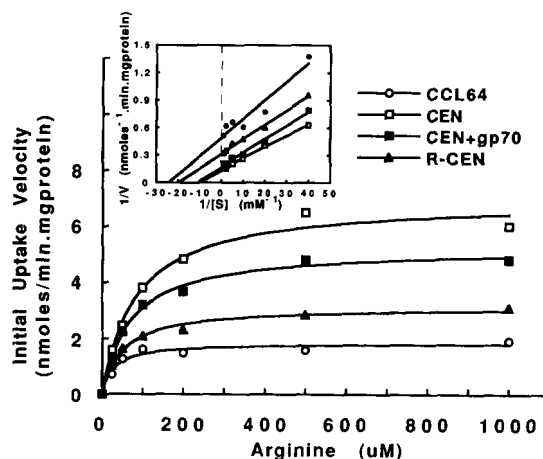


FIG. 3. Effect of gp70 adsorption and viral infection on L-[³H]arginine uptake into mink cells. CCL64 (○), CEN (□), CEN after being incubated with gp70 (4 μ g/ml) at 37 °C for 2 h (■), and CEN chronically infected with Rauscher MuLV (R-CEN) (▲) were exposed to different concentrations (25, 50, 100, 200, 500, and 1000 μ M) of radioactive arginine (specific activity 50 μ Ci/ μ mol) at 37 °C for 0.5 min. After two washings with ice-cold PBS, the radioactive arginine accumulated in the cells was extracted with 5% trichloroacetic acid and measured as described under "Materials and Methods." The lines were drawn by computer using least-squares fitting to the Michaelis-Menten equation. The inset is a double reciprocal plot of the same data. The computer-derived K_m values are $28 \pm 9 \mu$ M, $84 \pm 13 \mu$ M, $70 \pm 7 \mu$ M, and $51 \pm 6 \mu$ M for CCL64, CEN, CEN + gp70, and R-CEN cells, respectively. The corresponding V_{max} values for these same cells were 1.9 ± 0.1 , 7.0 ± 0.3 , 5.3 ± 0.1 , and 3.2 ± 0.1 nmol/min/mg of protein, respectively.

transport activity of the mink CCL64 cells provides a baseline for quantitative evaluation of the effects of infection or of gp70 adsorption.

To determine whether the partial loss of y^+ caused by infection with ecotropic MuLV is specific to mouse y^+ , we expanded the latter investigation (*i.e.* Fig. 3) to include analysis of mink CCL64 cells that were infected with ecotropic Rauscher MuLV. Because mink CCL64 cells lack ecotropic receptors, the infection was accomplished using virus that had been pseudotyped with an amphotropic host-range envelope (see "Materials and Methods"). Fig. 4 shows an immunofluorescence analysis that demonstrates expression of gp70 on the surfaces of mink CCL64 and CEN cells that were infected with Rauscher ecotropic MuLV. These mink cell lines were efficiently infected (greater than 99% positive for gp70), and they released virions that had the expected ecotropic host-range (data not shown).

Fig. 5 shows a comparison of L-[3 H]arginine uptake into

the uninfected and infected CCL64 cells and into the uninfected and infected CEN derivative cells that contain recombinant ecoR. Consistent with the previous data, infection of CEN cells caused substantial but incomplete loss of mouse y^+ uptake activity. However, infected and uninfected mink CCL64 cells had indistinguishable amino acid uptake curves, suggesting that ecotropic MuLV has no effect on mink y^+ . This strongly suggests that ecotropic MuLV infection causes partial down-modulation of mouse y^+ amino acid uptake activity, but has no effect on the endogenous mink y^+ transporter.

Fig. 6 presents evidence that the inhibitory effect of extracellular ecotropic gp70 on L-[3 H]arginine uptake is also specific to mouse y^+ . Thus, addition of a saturating amount of ecotropic gp70 at 37 °C reduced mouse y^+ import activity in CEN cells. Although this inhibitory effect of gp70 on CEN cells was small, it was reproducible and was determined to be statistically highly significant (see below). On the contrary,

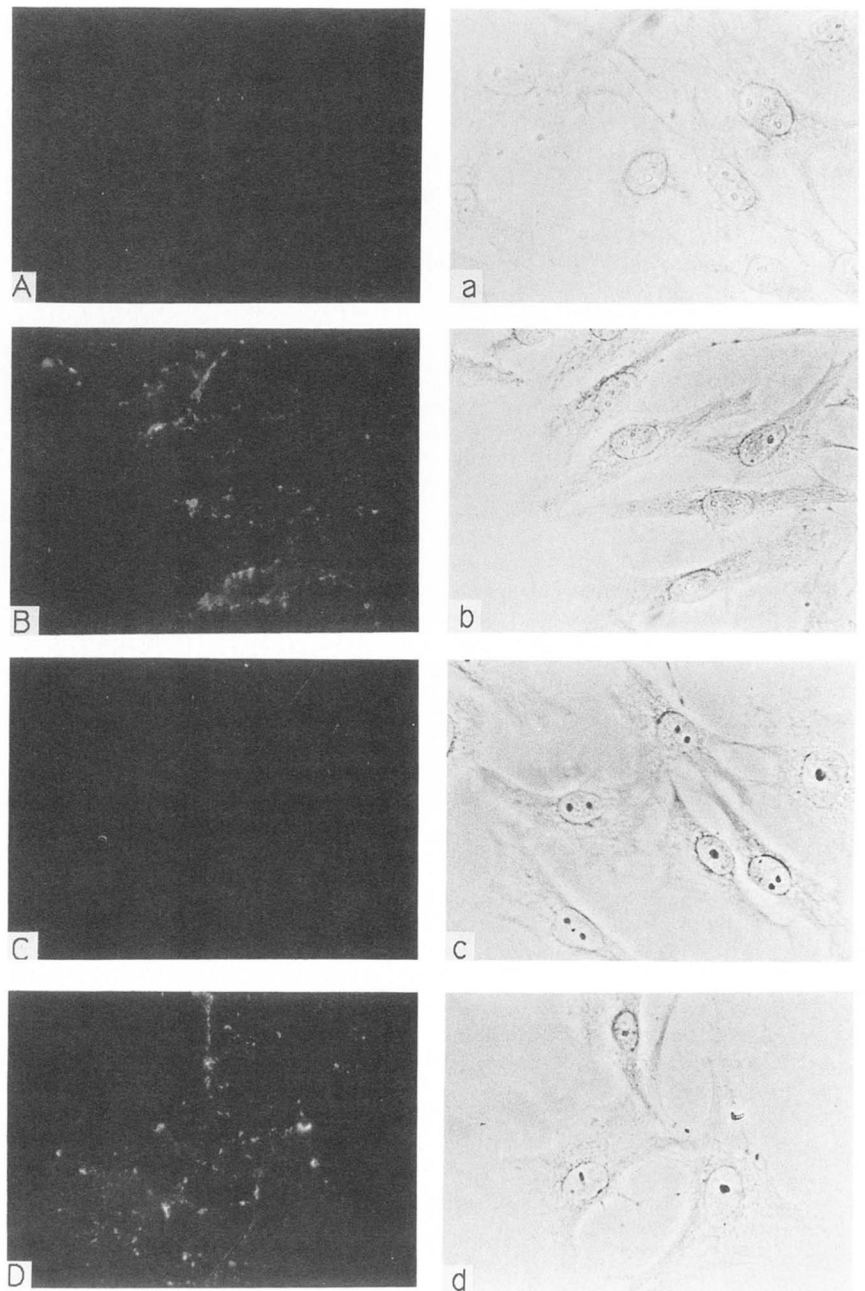


FIG. 4. Expression of gp70 on the surfaces of cells infected with ecotropic host-range Rauscher murine leukemia virus (R-MuLV). The immunofluorescence microscopy procedure is described under "Materials and Methods." Each microscopic field is shown in both fluorescence (*left panels*) and phase-contrast (*right panels*). *A, a*, mink CCL64 cells; *B, b*, mink CCL64 cells that had been infected with R-MuLV pseudotyped with an amphotropic envelope; *C, c*, CEN cells (these are CCL64 cells that express ecoR); and *D, d*, CEN cells chronically infected with R-MuLV.

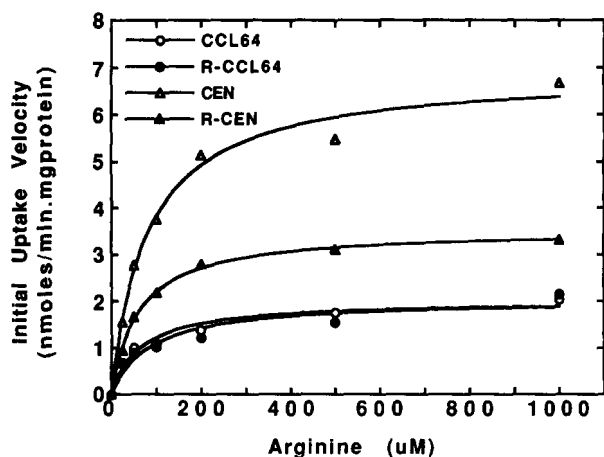


FIG. 5. Comparison of L-[³H]arginine uptake into mink CCL64 fibroblasts, CEN fibroblasts (mink CCL64 that express recombinant mouse *ecoR*), R-CEN (CEN infected with Rauscher ecotropic MuLV), and R-CCL64 (CCL64 cells that were infected with Rauscher ecotropic MuLV as described under "Materials and Methods"). Conditions for uptake studies are described in legend to Fig. 3 and under "Materials and Methods." The lines were drawn by computer using least-squares fitting to the Michaelis-Menten equation. The computer K_m estimates were $80 \pm 11 \mu\text{M}$, $60 \pm 0.4 \mu\text{M}$, $65 \pm 15 \mu\text{M}$, and $84 \pm 31 \mu\text{M}$ for CEN, R-CEN, CCL64, and R-CCL64 cells, respectively. The corresponding V_{max} estimates for these same cells were 6.9 ± 0.3 , 3.5 ± 0.1 , 1.9 ± 0.1 , and 2.0 ± 0.2 nmol/min/mg of protein, respectively.

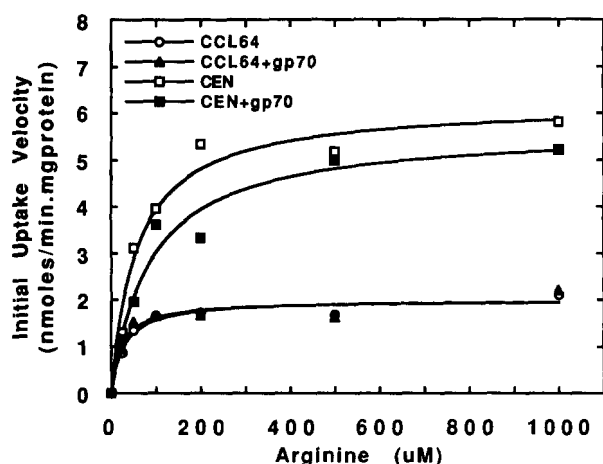


FIG. 6. Effect of ecotropic gp70 on L-[³H]arginine uptake into mink CCL64 fibroblasts and into CEN fibroblasts (CCL64 that express recombinant mouse *ecoR*), in the absence or presence of a saturating concentration of ecotropic gp70. Conditions for uptake studies were described in legend of Fig. 3 and under "Materials and Methods." Cells were incubated with purified gp70 (4 $\mu\text{g}/\text{ml}$) at 37 °C for 2 h prior to uptake studies. The lines were drawn by computer to the Michaelis-Menten equation. The computer K_m estimates were $57 \pm 13 \mu\text{M}$, $87 \pm 21 \mu\text{M}$, $27 \pm 7 \mu\text{M}$, and $22 \pm 8 \mu\text{M}$ for CEN, CEN + gp70, CCL64, and CCL64 + gp70, respectively. The corresponding V_{max} estimates for these same cells were 6.2 ± 0.4 , 5.7 ± 0.4 , 2.0 ± 0.1 , and 2.0 ± 0.1 nmol/min/mg of protein, respectively. Statistical analysis of many replicate experiments of gp70 effects are summarized in Table I.

gp70 had no effect on the endogenous mink transporter in the control CCL64 cells. Because ecotropic gp70 binds to mouse y^+ but not to mink y^+ (20, 21, 25), the inhibitory effect of gp70 must be caused by its binding to the transporter.

Table I summarizes results of many studies in which we measured effects of infection and of gp70 adsorption on L-[³H]arginine uptake into CCL64 mink fibroblasts and their CEN derivative that contains mouse *ecoR/y⁺*. These results

TABLE I
Effects of gp70 adsorption and viral infection on the V_{max} and K_m of arginine uptake in mink cells

Cells used	V_{max}^a	K_m^a	Number ^b
	nmol/min/mg of protein	μM	
CCL64	2.0 ± 0.1	60.4 ± 11.9	7
CEN	7.5 ± 0.8	97.0 ± 11.1	7
CEN + gp70	5.8 ± 0.4	91.1 ± 11.6	4
R-CEN	3.8 ± 0.3	80.0 ± 15.2	4

^a Values are presented as means \pm S.E.M. The V_{max} differences are significant by the paired comparisons *t* test (32) to at least 95% confidence for all of the cells indicated. The effect of gp70 on V_{max} of CEN cells is significant to 98% confidence. Reproducibly, the endogenous mink y^+ transporter of control CCL64 cells has a significantly lower K_m for arginine import than the mouse *ecoR/y⁺* transporter. The small apparent K_m differences between CEN, CEN + gp70, and R-CEN, while not significant to 95% confidence limits, are consistent with the lower K_m for mink y^+ and with the fact that these CEN cells contain an endogenous mink y^+ in addition to their different amounts of mouse y^+ . These mean K_m estimates differ somewhat from the values of the individual experiments reported in the legends of Figs. 3, 5, and 6.

^b Number of independent experiments.

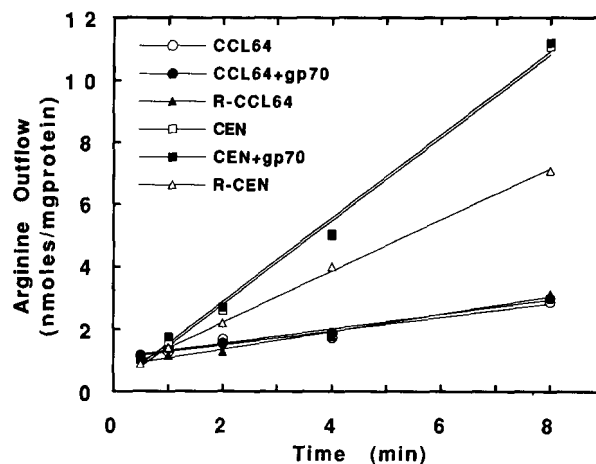


FIG. 7. Effect of gp70 adsorption and viral infection on L-[³H]arginine outflow from mink cells. Cells were preloaded with 200 μl of 1 mM L-[³H]arginine (50 $\mu\text{Ci}/\mu\text{mol}$) at 37 °C for 1.5 h. The radioactive arginine outside the cells was then quickly removed by washing three times with ice-cold EBSS, before 1 ml of EBSS at 37 °C was added to the cells. Samples of the media were taken at 0.5, 1, 2, 4, and 8 min with a multichannel pipette (Applied Scientific) while the cells were incubating at 37 °C. The CEN and CEN + gp70 cells release label rapidly; the R-CEN cells release label at an intermediate rate; and the CCL64, R-CCL64, and CCL64 + gp70 cells release the label at the slowest rate.

suggest that infection with Rauscher MuLV or adsorption of ecotropic gp70 at 37 °C significantly inhibit V_{max} for uptake by mouse y^+ with no significant effects on K_m values.

Analysis of L-[³H]arginine export from these mink cell lines is shown in Fig. 7. For export, cells were preloaded to equilibrium by incubation for 1.5 h with 1 mM L-[³H]arginine. Because system y^+ is electrogenic (2), it can mediate a substantial (10-fold, assuming a -60-mV resting potential) accumulation of substrate; such values are consistent with previous measurements in fibroblasts (4–7). This concentration is substantially above the K_m for export of this amino acid by system y^+ (approximately 0.8 mM) (5). These considerations predict that the rate of L-[³H]arginine release after removing extracellular arginine should be constant and equal to V_{max} for export for several minutes until the intracellular concentration declines toward the K_m . As expected, the initial rates

of export were constant (see Fig. 7). Moreover, the rate of export from CEN cells was substantially higher than from CCL64 cells, consistent with a larger amount of y^+ transporter in the CEN cells. The initial rate of export from ecotropic MuLV-infected R-CEN cells was considerably slower than from CEN cells but faster than from CCL64 cells. Thus, both import and export measurements suggest that there is less mouse y^+ transporter activity in the surface membranes of infected CEN cells. This reduction is specific to mouse y^+ because infected and uninfected mink CCL64 cells have the same quantity of L-[3 H]arginine transport activity. As with mouse Balb/c 3T3 cells (see Fig. 1), gp70 does not inhibit L-[3 H]arginine export from any of these cells. Moreover, this lack of effect of gp70 on outflow is true not only for the initial rate (*i.e.* V_{max} conditions) but also throughout the entire time course of outflow as the amino acid concentration approaches the intracellular K_m and then continues to decline toward zero (results not shown). This implies that intracellular K_m is also unaffected by gp70 binding to mouse y^+ .

Lack of Effect of Ecotropic MuLV on Proline Transport—As shown in Fig. 8A, L-[3 H]proline uptake into cells was identical in uninfected and Rauscher MuLV-infected mouse Balb/c 3T3 fibroblasts. Similarly, L-[3 H]proline uptake was not significantly different in mink CCL64, CEN, and Rauscher MuLV-infected CEN cells (Fig. 8B).

Highly Efficient Interference to Superinfection by Ecotropic Retroviruses in Both Mink and Mouse Cells—The above data

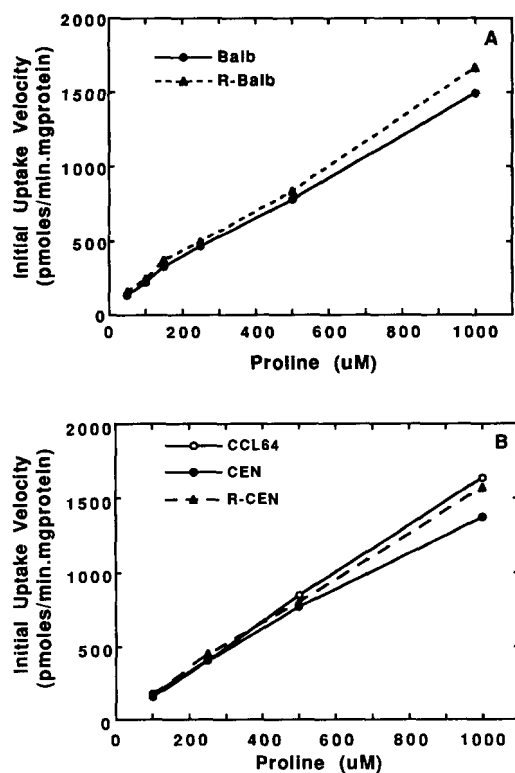


FIG. 8. Proline uptake into mouse and mink fibroblasts. Cell monolayers were exposed to different concentrations of L-[3 H]proline (50 μ Ci/ μ mol) for 1 min at 37 °C. After rapidly washing the cells twice with ice-cold PBS solution, the radioactivity in the cells was extracted with 5% trichloroacetic acid and measured in the liquid scintillation spectrophotometer as described under "Materials and Methods." *Panel A* shows initial rates of proline uptake in mouse Balb/c 3T3 fibroblasts and in Balb/c 3T3 fibroblasts chronically infected with Rauscher MuLV (*R-Balb*). *Panel B* shows the initial rates of proline uptake in mink CCL64 fibroblasts, CEN (mink CCL64 that express recombinant *ecoR*), and R-CEN (CEN infected with Rauscher MuLV).

indicate that infection of cells with ecotropic Rauscher MuLV causes only partial (2–3-fold) loss of cell surface mouse y^+ transporter, implying that *ecoR* must remain in a substantial amount on surfaces of infected cells. To determine whether the infected cells exhibit interference to superinfection, we attempted to superinfect them with an ecotropic host-range retrovirus that encodes human growth hormone and we analyzed the cells 48 h later for growth hormone-specific immunofluorescence. From the proportion of cells that were negative for growth hormone, and assuming the binomial distribution for virus infections in the cell populations, we calculated the multiplicities of infection (*i.e.* the average numbers of virus infections per cell) (see "Materials and Methods"). As shown in Table II, mouse Balb/c 3T3 and mink CEN that have *ecoR* were both highly susceptible to infection by the growth hormone-encoding virus, and high proportions of the cells expressed growth hormone. Cells that were preinfected with Rauscher ecotropic MuLV were completely resistant to superinfection with this growth hormone-encoding virus; no cells with growth hormone were detected among at least 1000 cells examined. However, cells infected with the virus of another host-range (MCF-13) remain susceptible to superinfection by the ecotropic growth hormone-encoding virus.

DISCUSSION

Infection with Ecotropic MuLV Causes Incomplete Removal of Mouse y^+ Activity from Cell Surfaces—These studies with mammalian cells confirm evidence obtained with *Xenopus* oocytes (1, 2) that mouse *ecoR* is a basic amino acid transporter with characteristics of system y^+ . Our results suggest that this mouse y^+ activity is partially down-modulated by chronic infection with ecotropic MuLV (see Figs. 1, 3, 5, and 7). This incomplete loss of transporter, detected as a reduction in V_{max} for both import and export of L-[3 H]arginine without any significant effect on K_m values, is specific to mouse y^+ and does not affect mink y^+ (Figs. 5 and 7 and Table I).

Down-modulation of mouse y^+ caused by infection with ecotropic MuLV is incomplete. This is seen in all of our studies as a residual mouse y^+ activity after efficient infection of both mouse and mink cells (Figs. 1, 3, 5, and 7 and Table I). In mink cells that express mouse *ecoR*, infection reduces y^+ activity to a residual level that is substantially higher than the baseline of endogenous transport activity that occurs in the control mink cells. Infection by ecotropic MuLV reduces

TABLE II

Infection of cells by an ecotropic virus that encodes human growth hormone

Cell type ^a	Multiplicity of infection ^b
Balb	1.48 \pm 0.13
R-Balb	<0.001 ^c
M-Balb	1.94 \pm 0.14
CEN	0.48 \pm 0.02
R-CEN	<0.001 ^c
M-CEN	0.41 \pm 0.04

^a Balb, R-Balb, and M-Balb are Balb/c 3T3 mouse fibroblasts that are uninfected, infected with ecotropic Rauscher MuLV, and infected with dualtropic MCF-13 virus, respectively. CEN, R-CEN, and M-CEN are CCL64 mink fibroblasts expressing *ecoR* that are uninfected, infected with ecotropic Rauscher MuLV, and infected with dualtropic MCF-13 virus, respectively.

^b Multiplicities of infection by the helper-free ecotropic host-range virus that encodes human growth hormone were determined by analysis of growth hormone-specific immunofluorescence (see "Materials and Methods"). Multiplicity of infection is the average number of infections that occurred per cell in the culture.

^c No positives were found among at least 1000 cells scanned.

mouse y^+ activity both for import and export of amino acids by approximately 50–70% in different experiments. Further understanding of this down-modulation would require direct studies of *ecoR* synthesis, processing, and turnover, and we are attempting to make antiserum for this purpose.

Implications for the Mechanism of Viral Interference—The partial loss of mouse *ecoR*/ y^+ from surfaces of cells infected with ecotropic MuLV cannot explain the essentially complete resistance of these cells to superinfection by ecotropic host-range retroviruses (e.g. see Table II). Indeed, we have presented evidence that CEN cells contain a vast excess of *ecoR*, which is not the limiting factor in infection (25). A 50–70% loss of *ecoR* would not be expected to reduce efficiency of infection.

Consequently, a mechanism other than loss of receptor must be responsible for viral interference in these cells. One possibility is that the *ecoR*/ y^+ on surfaces of infected cells is saturated with gp70 and therefore inaccessible to extracellular virus. According to this idea, interference is accomplished by blocking receptor sites on cell surfaces. A second possibility is that the *ecoR*/ y^+ remaining on surfaces of infected cells is nonfunctional in mediating superinfection because of a post-translational modification. For example, the third extracellular loop of *ecoR*, which is believed to contain the site for virus attachment, also contains potential sites for *N*-linked glycosylation (3). If these sites were incompletely glycosylated with heterogeneously structured oligosaccharides, as is common for glycoproteins (33), only a portion of mouse *ecoR*/ y^+ might be able to interact with gp70. Presumably, only this interactive portion would be eliminated by infection, and the remaining y^+ would be incapable of mediating superinfection. A third possibility is that infection may eliminate an accessory factor other than y^+ that is required for ecotropic viral entry into cells (25).

Inhibition of Mouse y^+ Transporter by gp70 Adsorption at 37 °C—In contrast to chronic infection, which may reduce receptor expression metabolically by affecting its processing and/or rate of degradation (10–13, 15), adsorption of a saturating amount of gp70 onto cell surface *ecoR* at 37 °C affects mouse y^+ transporter activity as an impermeant inhibitor. Specifically, gp70 significantly inhibits the V_{max} for import of L-[³H]arginine by mouse y^+ by approximately 25% without any significant effect on K_m (see Figs. 1, 3, and 6 and Table I). In contrast, gp70 has no significant effect on either V_{max} or K_m for export of arginine by mouse y^+ (see “Results”), and it has no effect on any parameter of mink y^+ transport. Because it is uncertain whether all mouse y^+ on uninfected cells is capable of binding gp70 (see above), we do not know the extent to which gp70 binding slows the mouse y^+ import cycle. Nevertheless, because gp70 binding has no significant effect on the export of arginine (Figs. 1 and 7), it is clear that gp70 is not an irreversible inhibitor that locks mouse y^+ into one conformational state. Therefore, *ecoR* with bound gp70 remains able to transport amino acids.

Generally, facilitated transport can be well described by a four-state model in which import operates in a clockwise direction and export in a counterclockwise direction (see Fig. 9A). In each cycle, transporter binds solute on one side, undergoes a conformational change that allows movement of solute through the membrane, releases the solute, and undergoes another conformational change that returns the empty transporter to its initial state (5, 34).

Previous workers have thoroughly analyzed kinetic properties of system y^+ (5–7). Like other transporters, the conformational changes of y^+ are several orders of magnitude slower than the rates of association and dissociation of solute. Indeed,

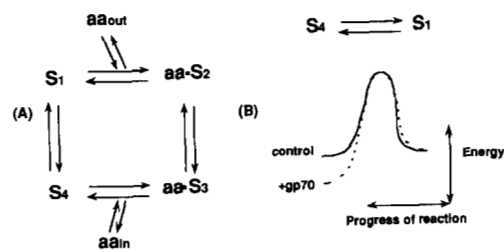


FIG. 9. Four-state model of y^+ transporter function and interpretation of gp70 inhibitory effects. A, the four-state model is shown. In the import cycle (clockwise), state S_1 binds extracellular amino acid to form the amino acid- S_2 complex. The transporter then changes conformation to form amino acid- S_3 from which the amino acid can dissociate into the cytoplasm to form the S_4 state. The final step in the import cycle occurs by conformational change of S_4 to regenerate S_1 . The amino acid export cycle operates in the counterclockwise direction. Substantial evidence (5–7) suggests that the $S_4 \rightarrow S_1$ transition is the rate-limiting step of the amino acid import cycle and that the opposite $S_1 \rightarrow S_4$ transition rate-limits the export cycle. This evidence derives in part from the existence of *trans*-stimulation. B, effects of gp70 binding are interpreted in terms of the energetics of the $S_4 \rightleftharpoons S_1$ transition, since these are believed to be the rate-limiting steps of the import and export cycles (see text). The basic interpretation of our data is that gp70 binding to *ecoR* stabilizes state S_4 and has relatively little effect on state S_1 . In the curve, the ordinate scale represents the potential energy, and the abscissa represents the progress of the reaction. Since by transition state theory the rate of any reaction is proportional to the probability of reaching the transition state and since this probability is related by the Boltzman distribution to the energy required (35), this model predicts that gp70 would inhibit the y^+ amino acid import cycle (i.e. $S_4 \rightarrow S_1$ transition) but have no effect on the y^+ export cycle (i.e. limited by the $S_1 \rightarrow S_4$ transition) (as observed).

by electrophysiological analyses, we have estimated that each y^+ maximally cycles in the import direction at approximately 200 times/s,² a rate typical for transporters, but orders of magnitude less than turnover numbers for many enzymes (34). Furthermore, evidence strongly suggests that the conformational change of the empty y^+ transporter (i.e. interconversion of states $S_4 \rightleftharpoons S_1$; see Fig. 9) is much slower than the conformational change that moves bound amino acid through the membrane (5–7). This evidence is based principally on the existence of “*trans*-stimulation,” a process whereby an unlabeled cationic amino acid on either side of the membrane greatly stimulates the rate of transport of a radioactive cationic amino acid from the opposite side. Moreover, such *trans*-stimulation of L-[³H]arginine uptake into CEN cells occurs both in the presence and absence of adsorbed gp70,³ strongly suggesting that the $S_4 \rightarrow S_1$ conformational change is rate-limiting for L-[³H]arginine import both in the presence and absence of gp70. Similar studies (5) have demonstrated that export of cationic amino acids by y^+ is limited by the opposite $S_1 \rightarrow S_4$ conformational change (see Fig. 9A).

Based on these considerations and on our results (Figs. 1, 3, 6, and 7), we propose the model for gp70 interaction with y^+ described in Fig. 9B. According to this model, gp70 adsorption onto mouse y^+ at 37 °C inhibits the rate-limiting $S_4 \rightarrow S_1$ step of the amino acid import cycle, but has no effect on the rate-limiting step of the export cycle (i.e. $S_1 \rightarrow S_4$ conversion). This can be readily understood from the perspective of transition state theory (35) by assuming that gp70 binds strongly to conformational state S_4 and only relatively weakly to state S_1 . This would increase the energy required to convert S_4 to the $S_4 \rightleftharpoons S_1$ transition state and would therefore inhibit uptake of amino acid. However, as shown in Fig. 9B, the

² M. Kavanaugh and R. A. North, unpublished results.

³ H. Wang and D. Kabat, unpublished results.

energetics of converting S_1 to the $S_4 \leftrightarrow S_1$ transition state would be unaltered by gp70, and the rate of export by y^+ would therefore be unaffected. This result occurs because the rate of $S_4 \rightarrow S_1$ conversion (or vice versa) depends on the transition state barrier that separates these states (35) and because we propose that gp70 heightens this barrier in the $S_4 \rightarrow S_1$ direction but not in the $S_1 \rightarrow S_4$ direction.

We conclude that ecotropic MuLVs bind to a conformationally mobile site on mouse y^+ . Indeed, this site with bound gp70 evidently continues to change conformation during amino acid transport at a rate of several hundred times per s. Conceivably, this could perturb stability of the viral membrane in a manner necessary for infection.

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