Carrier-mediated Transport of Oligopeptides in the Human Fibrosarcoma Cell Line HT1080

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ABSTRACT

To explore the feasibility of targeting human tumor cells via their transport systems, dipeptide uptake was studied in the human fibrosarcoma cell line HT1080 and the human fibroblast cell line IMR-90 by the use of hydrolysis-resistant glycylsarcosine (Gly-Sar). The uptake of [14C]Gly-Sar into HT1080 was time dependent. Kinetic analysis of the concentration dependence of the initial rate of [14C]Gly-Sar uptake showed that a carrier-mediated transport system with a \( K_m \) of 11.4 ± 3.3 mM and \( V_{max} \) of 26.8 ± 4.0 (nmol/15 min/mg protein) and a nonsaturable component \( (k_n) \) of 0.80 µL/15 min/mg protein were responsible for the dipeptide uptake by HT1080 cells. The optimal pH for the maximal uptake was around 6.0. [14C]Gly-Sar uptake was inhibited by various di- and tripeptides and peptide-mimetic drugs, such as bestatin and cefadroxil. [14C]Gly-Sar uptake was not affected by the presence of amino acids or tetra- or pentapeptides. The uptake of cefadroxil was reduced significantly by unlabeled Gly-Sar. Moreover, Gly-Gly and Gly-Leu produced an increase in the apparent \( K_m \) of the uptake of Gly-Sar without altering \( V_{max} \). On the other hand, dipeptide uptake by IMR-90, which is a normal diploid cell line (not malignant), showed no saturable transport.

INTRODUCTION

It is well known that tumor cells possess several transport systems to take up nutrients, including amino acids (1), nucleosides (2), glucose (3), and folate (4). Various studies on the delivery of anticancer drugs to tumor cells by using such transport systems have been reported. Melparan, which is a cancer-therapeutic, phenylalanine-conjugated nitrogen mustard, was taken up by L1210 cells via the amino acid transport system (5, 6). Moreover, a sugar-linked nitrogen mustard derivative has been developed as an anticancer drug (7). Methotrexate, an inhibitor of dihydrofolate reductase, is taken up in a concentrative manner by many tumor cells, such as leukemia cells of rodents and human, via the folate transporter (8, 9).

Interest in the transport of oligopeptides has been heightened by the recent cloning of a novel family of peptide-proton cotransporters. This family includes the rabbit small intestinal PepT1 (10) and the human and rat homologues of PepT1 (11—13), and a second member of the family, PepT2, in the kidney (14). However, there has been no report on the presence of an oligopeptide transporter in tumor cells. If such a transporter does exist in tumor cells, it should be possible to use it for the selective delivery of drugs to tumor cells because expression of PepTs is thought to be limited to epithelial cells in the small intestine and kidney (11, 14—16).

We have examined oligopeptide transport activity in the human fibrosarcoma cell line HT1080 (17) and the human fibroblast cell line IMR-90 (18) by using hydrolysis-resistant Gly-Sar. Here, we present the new finding that HT1080 cells have an oligopeptide transport activity exhibiting similar but not identical features to those of the well-characterized intestinal and renal dipeptide transporters.

MATERIALS AND METHODS

Materials. IMR-90 cells were purchased from JCRB (Osaka, Japan). RPMI 1640 and MEM were obtained from Nissui (Tokyo, Japan). HT1080 cells were prepared in our laboratories. FCS and nonessential amino acids were obtained from Life Technologies, Inc. (Grand Island, NY). t-Glutamine and Gly-Gly were obtained from Wako Pure Industries Ltd. (Osaka, Japan). Rat tail collagen (type I) was obtained from Collaborative Research Inc. (Bedford, MA). [γ-C]Gly-Sar (11.4 Ci/mmol) and [14C]inulin (210 MBq/g) were purchased from Amersham (Buckinghamshire, United Kingdom) and DuPont (Wilmington, DE), respectively. Cefadroxil was a gift from Bristol Banyu (Tokyo, Japan). Unlabeled Gly-Sar, Gly-Pro, Arg-Ala, bestatin, and amastatin were purchased from Sigma Chemical Co. (St. Louis, MO). The protein assay kit was purchased from Bio-Rad (Melville, NY). All other chemicals were commercial products of reagent grade. Human PepT1 cDNA was a gift from Dr. F. H. Leibach (Medical College of Georgia, GA).

Cell Cultures. HT1080 and IMR-90 cells were grown at 37°C in a 5% CO2,5% air atmosphere. HT1080 and IMR-90 cells were cultivated in RPMI 1640 culture medium and MEM containing 1% nonessential amino acids and 2 mM glutamine, each containing 10% FCS without addition of antibiotics, respectively (19). For the uptake study, HT1080 and IMR-90 cells were seeded at a density of 5.0 × 10⁴ cells/cm² and 1.0 × 10⁴ cells/cm², respectively, on multidishes (four wells) coated with collagen (20). HT1080 and IMR-90 cells were grown for 2 days and 7—8 days, and their passage numbers were in the ranges of 20—50 and 3—7, respectively.

Uptake Experiments. Uptake of [14C]Gly-Sar or cefadroxil by the cultured cells was examined at 37°C by the use of a reported method (21). The flux was measured in HBSS (0.952 mm CaCl₂, 5.36 mm KCl, 0.441 mm KH₂PO₄, 0.812 mm MgSO₄, 136.7 mm NaCl, 0.385 mm NaHPO₄, 25 mm D-glucose, and 10 mm MES or HEPES) adjusted to pH 6.0 with NaOH. The osmolality of the HBSS was maintained at 310 mosm/kg. To assess the effect of extracellular pH, the HBSS contained either 10 mM MES (pH 5.5—5.6) or 10 mM HEPES (pH 7.0 and 7.5) and was titrated to a desired pH with 5 mM NaOH. [14C]Inulin was used as a marker for the extracellular fluid that adhered to the cells during the washing procedure and to estimate the zero time for the nonspecific adsorption that is involved in the apparent uptake of test compounds. To quantify the amounts of [14C]Gly-Sar and [14C]inulin in the cells, the washed cells were solubilized by the addition of 5 mM NaOH (0.25 ml), followed by shaking for 2 h. The resultant lysates were neutralized with HCl and mixed with 4 ml of liquid scintillation cocktail, Clesso-l (Nacalai Tesque, Kyoto, Japan). Radioactivity was determined using a liquid scintillation counter (LSC-1000, Aloka Co. Ltd., Tokyo, Japan). The uptake of cefadroxil was determined by HPLC analysis. Fresh solutions of the test compounds were prepared for each experiment.

HPLC Analysis of [14C]Gly-Sar and Cefadroxil. [14C]Gly-Sar in the uptake medium was analyzed by HPLC for the initial 15 min. A 20-µl sample was separated on an analytical HPLC column, TSK gel ODS 80 T, 4.6 × 150 mm (Tosoh, Tokyo), using 0.2% trifluoroacetic acid solution as the mobile phase.
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Fig. 1A (left) shows that the uptake of 20 μM [14C]Gly-Sar by HT1080 cells was linear during the first 15 min (Fig. 1A, right). Steady-state uptake of the dipeptide was achieved within 120 min. In contrast, when the cells were incubated at 4°C (open circles), the uptake was very low and reached a plateau within 30 min, indicating that the binding to the cells was not responsible for the concentrative uptake observed at 37°C. Moreover, the cells at 37°C accumulated approximately three times more dipeptide than that observed at 4°C. These data suggest that [14C]Gly-Sar uptake occurs by carrier-mediated transport and that the dipeptide is apparently accumulated intracellularly. On the basis of the results obtained in Fig. 1A, the uptake rates were determined at 15 min in subsequent experiments.

To examine the dipeptide transport activity in normal diploid cells, dipeptide uptake was measured in the IMR-90 fibroblast cell line, which was established from human normal lung tissue. Fig. 1B shows the time courses of uptake of 20 μM [14C]Gly-Sar at 37 and 4°C and of 1.0 mg/ml [14C]inulin as a marker of extracellular fluid that adhered to the cells. Interestingly, there was no significant difference between dipeptide uptakes at 4 and 37°C within 120 min in IMR-90 cells. In addition, the extent of dipeptide uptake was comparable to that of inulin. These results suggest that no specialized transporter is involved in the [14C]Gly-Sar uptake in IMR-90 cells.

RESULTS

Time Course of [14C]Gly-Sar Uptake by HT108O and IMR-90 Cells. As a first step in the characterization of dipeptide uptake, the accumulation of [14C]Gly-Sar was examined at pH 6.0 in HT1080 and IMR-90 cells. Fig. 1A (left) shows that the uptake of 20 μM [14C]Gly-Sar by HT1080 cells was linear during the first 15 min (Fig. 1A, right). Steady-state uptake of the dipeptide was achieved within 120 min. In contrast, when the cells were incubated at 4°C (open circles), the uptake was very low and reached a plateau within 30 min, indicating that the binding to the cells was not responsible for the concentrative uptake observed at 37°C. Moreover, the cells at 37°C accumulated approximately three times more dipeptide than that observed at 4°C. These data suggest that [14C]Gly-Sar uptake occurs by carrier-mediated transport and that the dipeptide is apparently accumulated intracellularly. On the basis of the results obtained in Fig. 1A, the uptake rates were determined at 15 min in subsequent experiments.

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**Fig. 1.** Time courses of [14C]Gly-Sar uptake by HT1080 and IMR-90 cells. Uptakes of [14C]Gly-Sar (20 μM) by HT1080 cells (A) and IMR-90 cells (B) were measured at 37 (•) or 4°C (○) by incubating cells in HBSS at pH 6.0. △, uptake of [14C]inulin (1.0 mg/ml) in IMR-90 cells. Data points, means of four experiments; bars, SE.
Concentration Dependence of Gly-Sar Uptake. To examine the kinetics of Gly-Sar uptake by HT1080 cells, initial rates of Gly-Sar uptake were measured at pH 6.0 over the concentration range of 0.02–40 mM in the medium at 37 and 4°C to estimate total and nonsaturable uptake, respectively. As shown in Fig. 2, at 4°C the dipeptide uptake was linear over this concentration range. In contrast, when the cells were incubated at 37°C, the uptake rate tended to saturate as the Gly-Sar concentration increased. An Eadie-Hofstee plot of the uptake rates after correction for nonsaturable uptake gave no indication of multiple transport systems (inset), so the data were fitted to the following equation, which consists of single saturable and single nonsaturable linear terms, using the nonlinear least-squares regression analysis program, MULTI (23):

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} + k_d[S]. \]

The apparent kinetic constants obtained for Gly-Sar uptake were a half-saturation constant \(K_m\) of 11.4 ± 3.3 mM and a maximum uptake rate \(V_{\text{max}}\) of 26.8 ± 4.0 nmol/15 min/mg protein for the saturable component and a first-order rate constant \(k_d\) of 0.80 μL/15 min/mg protein for the apparently nonsaturable component.

pH Dependence of Uptake. The effect of varying the extracellular pH from 5.5 to 7.5 on the rate of \([^{14}C]\)Gly-Sar uptake was measured. As shown in Fig. 3, Gly-Sar uptake was influenced by the pH of the uptake medium. The optimal pH was about 6.0. This result implies that protons may be involved as the driving force for the concentrative transport of Gly-Sar in HT1080 cells.

Substrate Specificity of \([^{14}C]\)Gly-Sar. To study the substrate specificity of the transport system in cultured HT1080 cells, the uptake of Gly-Sar was determined in the presence of various compounds. As shown in Table 1, the uptake of the \([^{14}C]\)Gly-Sar was not influenced by free amino acids but was significantly reduced by various dipeptides. HPLC analysis confirmed that \([^{14}C]\)Gly-Sar was stable in the medium for the initial 15 min (data not shown). In the presence of 10 μM amastatin, an aminopeptidase inhibitor, uptake of Gly-Sar was not changed (data not shown). These observations indicate that the dipeptide was taken up as the intact molecule.

Arg-β-Ala, which contains a β-amino acid, was a less effective inhibitor than the peptide containing an α-amino acid (Arg-Ala). Furthermore, the inhibitory effect of Gly-Pro, which contains an imino acid, on \([^{14}C]\)Gly-Sar uptake was also weak. Gly-Sar, containing a methylated NH₂ terminus, was as inhibitory as Gly-Gly and Gly-Leu. The pseudodipeptide bestatin, an anticancer drug containing an NH₂-terminal α-hydroxy-β-amino acid, was clearly inhibitory. Cefadroxil, a β-lactam antibiotic drug having a tripeptide structure, is a substrate of the well-characterized intestinal and renal oligopeptide transporters, PepT1 and PepT2 (24–26).

Table 1 Inhibitory effects of various compounds on \([^{14}C]\)Gly-Sar uptake by cultured HT1080 cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Relative uptakeb (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>10</td>
<td>97.2 ± 7.3</td>
</tr>
<tr>
<td>Pro</td>
<td>10</td>
<td>112.5 ± 4.9</td>
</tr>
<tr>
<td>Gly-Gly</td>
<td>10</td>
<td>56.0 ± 4.7a</td>
</tr>
<tr>
<td>Gly-Leu</td>
<td>10</td>
<td>42.3 ± 4.8a</td>
</tr>
<tr>
<td>Gly-Sar</td>
<td>10</td>
<td>27.2 ± 8.3a</td>
</tr>
<tr>
<td>Gly-Pro</td>
<td>10</td>
<td>76.4 ± 4.8a</td>
</tr>
<tr>
<td>Arg-Ala</td>
<td>10</td>
<td>14.6 ± 0.8a</td>
</tr>
<tr>
<td>Arg-β-Ala</td>
<td>10</td>
<td>84.6 ± 5.2a</td>
</tr>
<tr>
<td>Bestatin</td>
<td>10</td>
<td>40.9 ± 5.3a</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>10</td>
<td>39.3 ± 4.9a</td>
</tr>
<tr>
<td>Gly-Gly-Gly</td>
<td>10</td>
<td>59.0 ± 7.2a</td>
</tr>
<tr>
<td>Gly-Gly-Gly-Gly</td>
<td>10</td>
<td>93.9 ± 2.1</td>
</tr>
<tr>
<td>Gly-Gly-Gly-Gly-Gly</td>
<td>10</td>
<td>98.4 ± 4.9</td>
</tr>
</tbody>
</table>

a The uptake of \([^{14}C]\)Gly-Sar (20 μM) was measured at 37°C for 15 min by incubating cells in HBSS buffer at pH 6.0 in the presence of each inhibitor. Each value represents % of control uptake and is shown as the mean ± SE of four experiments.
b Significantly different from the control value by Student's t test (P < 0.05).

Gly-Sar was not changed (data not shown). These observations indicate that the dipeptide was taken up as the intact molecule.

Fig. 2. Concentration dependencies of Gly-Sar uptake by HT1080 cells. Uptake of Gly-Sar was measured at 37°C (●) or 4°C (○) by incubating cells for 15 min with varied concentrations of Gly-Sar at pH 6.0. ▲, saturable component of the uptake rate calculated from the kinetic parameters, obtained as described in "Results." Inset, Eadie-Hofstee plot of the uptake rates after correction for the nonsaturable component. The line was drawn by the method of least-squares (correlation coefficient = 0.97). Data points, means of 3–8 experiments; bars, SE.
by cultured HT1080 cells in the absence and presence of 10 mM Gly-Leu (A). Incubation conditions were identical to those described in the Fig. 1 legend. Gly-Sar uptake was reduced by di- or tripeptides and peptide analogues, such as bestatin and cefadroxil, whereas amino acids or tetra- or pentapeptides had little or no effect (Table 1). In addition, Gly-Gly and Gly-Leu inhibited Gly-Sar uptake in a competitive manner (Fig. 4), and the uptake of cefadroxil was reduced in the presence of Gly-Sar (data not shown), showing mutual inhibition between them. These results strongly indicate that the HT1080 human fibrosarcoma cells take up oligopeptide and peptide-like drugs via a di- and tripeptide-specific, pH-dependent, carrier-mediated transport mechanism.

The peptide uptake observed in HT1080 cells exhibited pH dependency similar to that of the intestinal and renal PepTs, PepT1 and PepT2, indicating that a proton gradient is the driving force (31, 32). The substrate specificity of the transporter on HT1080 cells also exhibited close similarities with those of the well-characterized PepT1 and PepT2, which transport not only di- and tripeptides but also several peptide-mimetic drugs, such as β-lactam antibiotics and bestatin (25, 26, 33–35). Like PepT1 and PepT2, the transporter on HT1080 cells seems to accept peptide-mimetic drugs and a wide range of native peptides. However, in terms of the kinetic constant $K_m$ for the uptake of Gly-Sar, the PepT on HT1080 cells is different from PepT1 and PepT2. The apparent $K_m$ value of rabbit PepT1 obtained from the Xenopus oocyte expression system is 1.9 mM for Gly-Sar (10). The $K_m$s for Gly-Sar uptake by human PepT1 and PepT2 cDNA-transfected HeLa cells are 290 μM (11) and 74 μM, respectively (36). PepT2 is a high-affinity proton/peptide cotransporter, in contrast to PepT1. We found that the $K_m$ value of the transporter on HT1080 for Gly-Sar was 11.4 mM, indicating that the affinity is much lower than those of PepT1 and PepT2. Furthermore Northern blot analysis using human PepT1 cDNA as the probe did not show a corresponding signal in HT1080 cells. Taken together, the results indicate that the oligopeptide transporter expressed in HT1080 cells is a novel transporter, distinct from PepT1 and PepT2.

Interestingly, the present study indicated that Gly-Sar was not actively transported in IMR-90 cells, which are normal diploid cells from human lung connective tissue, suggesting that a dipeptide transporter exists in malignant cells, but not in normal diploid cells. This is important from the viewpoint of targeting anticancer drugs because the transporter becomes a good candidate for tumor cell-specific delivery of oligopeptide-mimetic anticancer drugs.

In conclusion, we have demonstrated the existence of an oligopeptide transporter on HT1080 cells, and we showed that Gly-Sar transport by this transporter is sensitive to pH and temperature and is saturable. Furthermore, the substrate specificity is very similar but not identical to those of well-characterized PepT1 and PepT2. Molecular identification of the oligopeptide transporter on HT1080 cells and confirmation of its selective expression in various tumor cells but not normal cells will be necessary to validate the feasibility of using this transporter for tumor-specific drug delivery.

REFERENCES

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