Combinatorial Targeting of the Macropinocytotic Pathway in Leukemia and Lymphoma Cells*

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Ligand-directed delivery of agents to leukemia and lymphoma cells has the potential to yield new mechanistic disease insights and targeted therapies. Here we set out to target the macropinocytotic pathway with a combinatorial approach. From the screening of acute T-lymphoblastic leukemia Molt-4 cells with a random phage-display peptide library, we isolated a phage displaying the sequence CAYHRLRRC. This peptide contains a lymph node-homing motif (Cys-Ala-Tyr) and a cell-penetrating motif (Arg-Leu-Arg-Arg). Binding of this ligand-directed phage to a large panel of leukemia/lymphoma cells and to patient-derived samples was much higher than to non-leukemia control cells. CAYHRLRRC phage internalization into Molt-4 cells is both energy- and temperature-dependent. Flow cytometry with fluorescein-labeled peptide and endocytosis blocking with specific inhibitors revealed that CAYHRLRRC is indeed taken up through macropinocytosis in Molt-4 and K562 human leukemia cells. Unexpectedly, the cell surface receptor for the CAYHRLRRC peptide is not a heparan sulfate proteoglycan as it would be predicted for other cell-penetrating peptides. Confirming this interpretation, a CAYHRLRRC-directed peptidomimetic-induced cell death in all the leukemia and lymphoma cells was evaluated, whereas a control transactivator of transcription protein (tat)-directed proapoptotic peptidomimetic was non-selective. In summary, the targeting peptide CAYHRLRRC is selectively internalized through macropinocytosis in leukemia and lymphoma cells and has potential as a drug lead for ligand-directed anti-leukemia therapies.

Leukemias and lymphomas are hematological malignant diseases characterized by impaired differentiation, increased clonal cell proliferation, and hematopoiesis suppression; the standard treatment for these tumors today is still predominantly based on nonspecific cytotoxics that disrupt nucleic acid and protein synthesis, often with severe side effects and relatively poor outcomes (1–3). However, selective anti-leukemia drugs have recently been developed (4), thus conceptually validating the scientific hope for a revolutionary targeted pharmacology against this group of diseases.

Over the past decade, we have selected phage-display random peptide libraries in vitro and in vivo to isolate and exploit tumor-specific and angiogenesis-related ligand-receptor systems toward targeted drug design and translation (5–7). Because cell trafficking and homing from the blood and/or lymphatic vessels to lymphoid and myeloid tissues to virtually all organs are essential leukocyte functions, we reasoned that targeting membranes would be a suitable approach to discover leukemia-specific ligands. Cell surface-binding peptide motifs have been reported in lymphoma and leukemia lines (8–11). Unfortunately, so far their corresponding receptors are either unknown (9, 10) or relatively nonspecific adhesion molecules such as certain integrins (8, 11) to which ligand binding does not enable clear enough differentiation between normal leukocytes and tumor cells; as a result, potentially useful ligand-receptor systems have not as yet emerged in leukemias and lymphomas. Thus, rather than attempt to identify other ligand (peptide)-receptor (protein) systems in leukemia- or lymphoma-derived cells, we reasoned that targeting a physiological cell translocation mechanism may serve as an alternative approach to this challenge.

As a proof-concept, here we selected cell-penetrating peptides that target the macropinocytotic pathway. Macropinocytosis has recently been recognized as a major pathway for the endocytic uptake of arginine-rich peptides and their protein conjugates (12–14) relative to other internalization mechanisms such as clathrin-dependent endocytosis (15, 16) or energy-independent, direct membrane translocation (17). Indeed, macropinocytosis is mechanistically different from pinocytosis pathways, such as clathrin-mediated, caveola-dependent, and clathrin- and caveola-independent endocytosis (18–20).

In the present study, we demonstrate the selection of cell-penetrating peptides that target the macropinocytotic pathway. In the dominant cell-penetrating peptide selected in one of the screenings, we also found a secondary motif likely responsible for the observed leukemia/lymphoma cell specificity. Finally, we functionally evaluated our cell-penetrating peptide prototype in its ability to specifically deliver a
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The abbreviations used are: FITC, fluorescein isothiocyanate; ALL, acute lymphoma-derived cells, relative to a panel of adherent tumor cells and normal leukocytes. Together, these results define a new combinatorial strategy for macropinocytosis-based studies with potential for targeted drug delivery to human leukemia and lymphoma cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Methyl β-cyclodextrin, chlorpromazine, wortmannin, 5-(N-ethyl-N-isopropyl)amiloride, propidium iodide, sodium heparin, heparanase III, fluorescein isothiocyanate-labeled transferrin (FTTC-Tf), and rabbit anti-Fd bacteriophage antibody were purchased from Sigma. 5-Carboxyfluorescein and 5-carboxyfluorescein tetramethylrhodamine were from Novabiochem (Darmstadt, Germany). Alexa Fluor 488- or 594-conjugated goat anti-rabbit IgG and TRITC-labeled neutral dextran 70 kDa (ND70) (TRITC-ND70) were purchased from Molecular Probes (Eugene, OR). BD Cell-TAK was from BD Biosciences. The WST-8 cell counting kit was from Dojin Medical Chemical Co. (Kumamoto, Japan). All the peptide synthetic reagents were from Applied Biosystems, Novabiochem, and Watanabe Chemical Co. (Hirosima, Japan).

**Cell Lines**—Leukemia and lymphoma cell lines or adherent non-leukemia tumor cells were maintained in RPMI 1640 or Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum, 20 μg/ml penicillin, and 20 μg/ml streptomycin, in 5% CO₂. The leukemia and lymphoma cells were obtained as follows: Molt-4, CCRF-CEM, Jurkat-T, T-ALL1, HL-60, and U937, Health Science Research Resources Bank (HSRRB), Osaka, Japan; HPB-ALL, RPMI-8226, THP-1, and K562, Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan; and SR-796 and TUR, American Type Culture Collection, Manassas, VA. Adherent non-leukemia cell lines were from the following suppliers: HepG2, HEK293, MCF-7, and A549, Riken Cell Bank, Wako, Japan; PC-3, WM115, U251MG; and DAOY, HSRRB; and PC-9, Immuno-Biological Laboratories Co., Takasaki, Japan.

**Peptide-display Phage Library Screening**—A phage-display random peptide library based on an fUSE5-derived vector displaying an insert with the general arrangement CX7C (C, cysteine; X, any residue) was designed and constructed with a diversity of 10⁴ to 10⁹ unique sequences (21). We used the bio-panning and rapid analysis of selective interactive ligands (termed BRASIL) methodology for the screening of cell-binding phage (22–24). In each screening, human leukemia Molt-4 cells were collected, washed in phosphate-buffered saline (PBS) containing 2.5 mM EDTA, and re-suspended in RPMI 1640 medium containing 1% BSA (BRASIL binding buffer) at 1 x 10⁴ cells per ml. The cell suspension (200 μl) was incubated with 10⁹ TU of the CX7C phage library or of each phage at 4 °C. After 2 h, the phage/cell mixture (aqueous phase) was gently transferred to the top of a non-miscible organic phase (200 μl/tube) consisting of dibutyl phthalate/cyclohexane (9:1 (v/v), p = 1.03 g ml⁻¹) and centrifuged at 10,000 x g for 10 min at 4 °C. The tube was quickly frozen in liquid nitrogen, the bottom of the tube was sliced-off, and the cell-phage pellet was isolated. The cell-bound phages were recovered by infection of host bacteria (K91/kan). Around rounds 2, 3, and 4, isolated bacteria cells from single clones were grown as individual colonies, and inserts from randomly picked clones were sequenced with the primer 5’-CCCTCATAGTTAGCGTAACGATCT-3’.

**Peptide Synthesis**—Peptides were synthesized by using Fmoc-(N-(9-fluorenyl)methoxycarbonyl) chemistry on a Pioneer peptide synthesizer (Applied Biosystems Japan Inc., Tokyo, Japan) and purified by reverse-phase high-performance liquid chromatography to >95%. Identification of the peptides was carried out by mass analysis using matrix-assisted laser desorption time-of-flight mass spectrometry. Peptides containing two cysteine residues derived from the CX7C library were cyclized by air oxidation prior to purification. Fluorescein-labeled peptides were synthesized by introduction of 5-carboxyfluorescein or 5-carboxyfluorescein tetramethylrhodamine to the N terminus at the final synthetic cycle. The abbreviations and sequence of each of the synthetic peptides were as follows: CAYHRLRCC-GG; A-CAYHRLRCC-GG; SAYHRLRRS, A-SAYHRLRRS-GG; CAYHALAAC, A-CAYHALAAC-GG; CAYHALAAC-GG-(KLAKLAK)₂, A-CAYHRLRCC-GG-GG-(KLAKLAK)₂, t tat-(KLAKLAK)₂, G-GRKRRQQKRRR-GG-(KLAKLAK)₂, fam-CAYHALAAC, 5-carboxyfluorescein-A-CAYHRLRCC-GG; tamra-CAYHALAAC, 5-tetramethylcarboxyfluorescein-A-CAYHRLRCC-GG; fam-tat, 5-carboxyfluorescein-GG-GRKRRQQKRRR-GG. The extra residues Ala (at the N terminus) and Gly-Gly (at the C terminus) of the CX7C sequence were added as a phage sequence-derived residue and a spacer, respectively. Gly-Gly at the N terminus and Gly-Gly-Cys at the C terminus of tat were also added as spacers to prevent steric hindrance.

**Phage Binding Assay**—Phage binding assays were performed as described (22, 23). A total of 10⁶ cells were incubated in the BRASIL binding buffer with 10⁷ TU of the CAYHRLRCC phage or fd-tet phage at 4 °C for 2 h. Cell-bound phage was separated from unbound phage by BRASIL selection and was recovered by infecting the host bacteria (K91/kan) followed by counting the bacterial colonies at serial dilutions. Insertless fd-tet phage served as a negative control. The CAYHRLRCC phage binding to the cells is shown relative to that of the fd-tet phage (which was set to 1). Competition assays were performed with or without the competitor under the same conditions. For removal of cell surface proteins, the cells were pretreated with 0.1% (w/v) trypsin, serum-free medium for 10 min at 37 °C. Trypsin was subsequently inhibited by the addition of 1 volume of fetal bovine serum, and the cells were washed with BRASIL buffer prior to use in the binding assay.

**Phage Internalization Assays**—We performed an internalization assay combined with phage binding. 10⁶ cells were incubated with 10⁹ TU of CAYHRLRCC phage for 1 h at 37 or 4 °C. Subsequently, cells were treated in fetal bovine serum-free RPMI 1640 containing 0.1% (w/v) trypsin medium to remove
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cell surface-bound, non-internalized phage. After washes with PBS, cells were either lysed with hypotonic buffer (2 mM Tris-HCl, pH 7.4) or not lysed (control). The residual titer was quantified by addition of Kanamycin-resistant Escherichia coli K91/kan to the cell lysate or intact cells. Recovery of the phage was shown relative to each non-lysed phage, which was incubated at 4 °C (which was set to 1).

Alternatively, fluorescent microscopic analysis was carried out as described (25). 10^6 cells were incubated with CAYHRLRRC phage (10^9 TU) at 37 °C for 2 h. Cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, and incubated with 0.2% Triton X-100, and blocked with 1% BSA in PBS. Cells were next incubated with a rabbit anti-fluorescein antibody rabbit secondary antibody (diluted 1:500 in PBS containing 1% BSA for 1 h at room temperature. After washes with PBS, cells were fixed on coverslips by air-drying. Phage particles were observed with a fluorescence microscope (Olympus FV3000, Tokyo, Japan; Keyence Biozero, Tokyo, Japan) equipped with a ×40 objective lens. Control fd-tet phage or FITC-labeled transferrin (5 μM) was used as a negative control or clathrin-dependent endocytosis marker, respectively. Nuclei were stained with Hoechst 33342 (2.5 μg/mL).

Peptide Internalization Assay—Internalization of the peptide was analyzed by flow cytometry on living cells (15). Leukemia Molt-4 and K562 cells were treated with the fluorescein-labeled peptide (fam-CAYHRLRRC or fam-tat) at various concentrations in serum-free RPMI 1640 medium at the indicated temperature and times. Cells were treated with 1% (v/v) trypsin for 10 min to remove extracellular bound proteins and peptides. After incubation, 1 volume of calf serum was added to inhibit the enzyme, the cells were washed with cold Hanks’ balanced salt solution three times, and the internalized peptides were analyzed by flow cytometry on a FACSscan fluorescence-activated cell sorter (BD Biosciences). A minimum of 10,000 cells/sample was analyzed by gating on live cells and exclusion of propidium iodide-labeled dead cells. For depletion of cellular debris, cells/assay was analyzed by gating on live cells and exclusion of activated cell sorter (BD Biosciences). A minimum of 10,000 events were analyzed by flow cytometry on a FACScan fluorescence-activated cell sorter (BD Biosciences). A minimum of 10,000 events were analyzed by flow cytometry on a FACScan fluorescence-activated cell sorter (BD Biosciences).

RESULTS

Isolation Phage Selective for Leukemic Cells—A phage-display random peptide library CX7C (7-mer peptides bounded by cysteine at their N and C termini) was screened on an acute T-lymphoblastic leukemia cell line, Molt-4, with the BRASIL method (22). We performed phage selection in excess of a competing Arg-Gly-Asp (RGD) synthetic integrin-binding peptide to minimize the recovery of RGD-containing peptides (23). After the 2 to 4 rounds of serial selection, individual clones (n = 96) were recovered from the Molt-4 cell lines and sequenced. We looked for arginine-rich motifs reminiscent of cell-penetrating peptides (29–31). By the end of the selection, 57% of the clones displayed peptide CAYHRLRRC (Table 1). Notably, the amino acid sequence of this dominant peptide was composed of a chimera of one such arginine-rich motif (Arg-Leu-Arg-Arg) commonly found in cell-penetrating peptides.
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The CAYHRLRRC Phage Bind to Leukemia/Lymphoma Cell Lines—We tested whether the CAYHRLRRC phage bound to a panel of leukemia/lymphoma or non-leukemia cells at 4 °C. Compared with insertless fd-tet phage (negative control), the CAYHRLRRC phage bound much more to Molt-4 cells (Fig. 1). Indeed, the CAYHRLRRC phage bound more to all of the T-ALL cells (CCRF-CEM, Jurkat-T, TALL-1, and HPB-ALL) over control. Moreover, the CAYHRLRRC phage also bound to myeloma RPMI-8226, erythroid-megakaryocytic K562, acute myeloid HL-60, and monocytic THP-1 as well. Finally, the CAYHRLRRC phage bound to human lymphoma cells such as SR-786, U937, and TUR. Together, these data indicate that the CAYHRLRRC phage bind preferentially to all leukemia-derived and lymphoma-derived cell lines tested. In contrast, binding to a panel of assorted non-leukemia tumor cell lines such as A549, MCF-7, and HepG2 occurred at levels similar to that of negative control phage (Fig. 1). CAYHRLRRC phage therefore showed a significant selectivity of binding to leukemia/lymphoma cell lines grown in suspension rather than to adherent, non-leukemia lines.

We next tested whether the CAYHRLRRC phage bound to normal leukocytes or primary tumor cells cultured from a representative panel of leukemia patients. In 10 of 13 clinical samples (77%), CAYHRLRRC phage bound more than twice relative to the fd-tet phage; binding of CAYHRLRRC phage to 3 leukemia samples was similar to control as it was binding to human bone marrow-derived cells, T-cell lymphocytes, monocytes, and M2-type macrophages from healthy peripheral blood (Table 2). Collectively, these results indicate that the selected chimeric (dual motif) peptide CAYHRLRRC has potential to target leukemia in translational applications.

Arginine Residues in the CAYHRLRRC Peptide Are Critical for Binding—To identify the residues of the CAYHRLRRC peptide that are required for phage binding, we carried out competition assays with synthetic peptides. Binding of the CAYHRLRRC phage to Molt-4 cells was inhibited by the corresponding synthetic CAYHRLRRC peptide in a concentration-dependent manner (Fig. 2A; IC50 ~ 20 μM). A linear peptide analog SAY-HLRRS, in which the terminal two cysteines were changed to serines, also inhibited phage binding in vitro. In contrast, a synthetic peptide in which the three Arg residues were changed into Ala residues failed to inhibit phage binding. These results indicate that secondary structure via the disulfide bridge of the peptide is not absolutely required for targeting in vitro; a result not at all inconsistent with the aggregate of our own in vivo observations (32). Moreover, the data confirm that Arg residues are crucial for binding of the phage to the leukemia cells, a behavior that would be expected from cell-penetrating peptides. Finally, we also observed a decrease in CAYHRLRRC phage binding to Molt-4 cells after exposure of the cells to trypsin at 37 °C but not at 4 °C (Fig. 2B), a result again indicating

![Figure 1. CAYHRLRRC phage targets leukemia and lymphoma cells. Binding of the CAYHRLRRC phage to a panel of leukemia/lymphoma or non-leukemia cells was evaluated by a phage binding assay, with quantification of TU in E. coli/H11006. Phage (10^7 TU) were incubated for 2 h at 4 °C on 10^6 cells, and the bound phage were separated from unbound phage by the BRASIL method. Phage (10^7 TU) were incubated for 2 h at 4 °C on 10^6 cells, and the number of bound phage was measured after separating from unbound phage. CAYHRLRRC phage binding to the cells is shown relative to that of fd-tet phage, for which the value was set to 1. Results are expressed as mean ± S.E.](image-url)

| Patient No. | Leukemia subclass (FAB classification)* | Phage bindinga (fold vs. fd-tet) |
|-------------|----------------------------------------|---------------------------------|
| 1           | L2                                    | 1.5                             |
| 2           | L2                                    | 1.5                             |
| 3           | L2                                    | 1.5                             |
| 4           | L2                                    | 1.5                             |
| 5           | L3                                    | 5.8                             |
| 6           | L3                                    | 2.1                             |
| 7           | L3                                    | 5.7                             |
| 8           | M2                                    | 0.7                             |
| 9           | M2                                    | 6.9                             |
| 10          | M2                                    | 2.4                             |
| 11          | M4                                    | 14.1                            |
| 12          | M4                                    | 2.0                             |
| 13          | M5                                    | 4.1                             |
| BM-derived Cells | Normal     | 0.4                             |
| T-lymphocytes | Normal     | 1.2                             |
| Monocytes   | Normal     | 1.1                             |
| M2-type macrophages | Normal | 0.9                             |

a FAB, French-American-British.
b Binding of the CAYHRLRRC phage to primary mononuclear cells either from patients with leukemia or from normal (healthy) volunteers was evaluated by the BRASIL method. Phage (10^7 TU) were incubated for 2 h at 4 °C on 10^6 cells, and the number of bound phage was measured after separating from unbound phage. CAYHRLRRC phage binding to the cells is shown relative to that of fd-tet phage (negative control), for which the value was set to 1.

BM, bone marrow.
that CAYHRLRRC may home differently under a harsh proteolytic in vivo environment (5–7, 32).

The CAYHRLRRC Phage Is Internalized by the Cells—We have previously demonstrated that some tumor-homing peptides isolated by in vivo and in vitro phage display were internalized upon binding to the target cells (25, 33–37). By using two methods, we therefore asked whether CAYHRLRRC phage were internalized by human leukemia Molt-4 and K562 cells as compared with normal leukocytes from healthy volunteers. In the first method, internalized CAYHRLRRC phage were recovered and determined as follows: leukemia Molt-4 or K562 cells were incubated with CAYHRLRRC phage for 2 h at 37 or 4 °C, and cell surface-bound phage were removed by incubation with trypsin. The internalized phage were recovered by cell lysis and then quantified. Consistently, the cells that were incubated at 37 °C, prior to lysis, produced the highest numbers of recovered phage (Fig. 3, A and B). The phage recovered from cells that were either incubated at 4 °C or were not lysed, showed levels similar to those from control fd-tet phage, data indicating that internalized phage were not detected. In contrast to human leukemia cells, normal T-cell lymphocytes exhibited no significant uptake of the CAYHRLRRC phage compared with insertless fd-tet phage (Fig. 3C). M2-type macrophages as an additional control, however, markedly endocytosed both phage clones in a temperature-dependent manner (Fig. 3D). In fact, and consistently with this result, it has been reported that M2-type macrophages differentiated from monocytes with macrophage colony stimulating factor have the inherent ability to take up almost any soluble molecules (including BSA and low density lipoprotein) through a receptor-independent mechanism (27, 38).

A second method used to evaluate phage internalization was immunofluorescence microscopy. CAYHRLRRC phage was internalized into both human leukemia cell lines (Molt-4 and K562) and observed as several small vesicles, whereas only background fluorescence was observed when insertless control fd-tet phage or no phage were used (Fig. 3E). In contrast, we did not observe any fluorescent particles of CAAYHRLRRC phage in normal T-lymphocytes. However, by using M2 macrophages as an additional control cell type, phage particles were detected, even when insertless control phage were used. Whereas these data indicate that the dominant mechanism of uptake of CAYHRLRRC-targeted phage by M2 macrophages is non-selective, one cannot rule out the possibility that a receptor-mediated endocytosis may also occur but is masked by the net cell internalization effect. Taken together, it appears that the CAYHRLRRC phage is preferentially internalized into leukemia cells upon ligand binding.

Localization of the internalized CAYHRLRRC phage in Molt-4 cells was compared with that of fluorescein-labeled transferrin, which is internalized through clathrin-mediated endocytosis. No co-localization of the internalized phage with transferrin was observed (Fig. 3F). Together, these results indicate that the endocytotic pathway that utilized the CAYHRLRRC phage is different from that mediated by clathrin.

The Synthetic CAYHRLRRC Peptide Is Internalized Outside of the Phage Context—To begin to evaluate whether the CAYHRLRRC peptide could ultimately serve as a drug carrier to leukemia or lymphoma cells, we synthesized fluorescein-labeled peptide (fam-CAYHRLRRC) and examined its internalization activity by flow cytometry. Marked uptake of fam-CAYHRLRRC peptide by Molt-4 cells was observed at 37 °C, but not at 4 °C (Fig. 4, A and B). Pre-treatment of Molt-4 cells with metabolic inhibitors such sodium azide and 2-deoxyglucose (to deplete ATP pools) decreased the uptake of the soluble peptide to 60% of control. These observations indicate that uptake of the peptide is energy-dependent and are consistent with results from the phage internalization assay (Fig. 3, A and E). Uptake of fam-CAYHRLRRC peptide was time- and dose-dependent (Fig. 4, C and D). These data support the claim that endocytosis is a major pathway for the uptake of the CAYHRLRRC peptide as well.

Uptake of the CAYHRLRRC Peptide Occurs via Macropinocytosis—To elucidate the mechanism by which the CAYHRLRRC peptide is internalized into Molt-4 cells, we examined effects of several types of endocytosis inhibitors on the uptake of fam-CAYHRLRRC peptide. The macropinocytosis inhibitors 5-(N-ethyl-N-isopropyl)amiloride and wortmannin significantly inhibited internalization of the fam-CAYHRLRRC peptide.
LRRC peptide (35 and 20% of control, respectively; Fig. 5A). Methyl β-cyclodextrin (MβCD) was also inhibited, to a lesser degree (48%). In contrast, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, did not suppress the uptake. MβCD is a lipid raft-dependent endocytosis inhibitor that depletes membrane cholesterol, but it is also known to inhibit macropinocytosis (19). From biochemical evidence that lymphatic cells such as Molt-4 cells lack cholesterol-rich caveola (39), the lipid raft-dependent pathway appears not to be involved in cellular uptake of the peptide. MβCD therefore has clearly inhibited the macropinocytic pathway in Molt-4 cells. In parallel experimentation with the human myelogenous leukemia K562 cells, uptake of fam-CAYHRLRRC was also markedly inhibited by 5-([N-ethyl-N-isopropyl]amiloride, wortmannin, and MβCD, but not by chlorpromazin (Fig. 5B). Notably, the effect of macropinocytosis inhibitors on K562 cells was somewhat lower than that for lymphoblastic Molt-4 cells. This result may perhaps be explained by an alternative endocytic pathway such as direct translocation, which has been reported in myeloid KG1a cells (17), that might exist in the K562 line because it shares similar cell origins.

ND70 is a specific fluid phase marker that is used to trace internalization via macropinocytosis, whereas transferrin is used as a clathrin-mediated marker of endocytosis. When we examined the intracellular localization of the CAYHRLRRC phage by confocal microscopy, most appeared coincident with ND70, but not with transferrin (Fig. 5C). Bound CAYHRLRRC peptide is therefore internalized by Molt-4 cells largely via macropinocytosis.

The CAYHRLRRC Peptide Internalization Mechanism Differs from That of tat—The human immunodeficiency virus-1 (HIV-1) tat-derived peptide (tat) is a small basic peptide that translocates into live cells as a cell-penetrating peptide. The sequence of tat contains several Arg residues that are critical for internalization (14, 31). A recent study proposed that macropinocytosis is one of the major pathways for the endocytic uptake of tat peptide and its protein conjugates (12–14), although the entry mechanism is controversial (40). Cellular uptake of tat peptide is highly dependent on heparan sulfate proteoglycans (HSPGs) (41). Because the CAYHRLRRC peptide also contains three Arg residues that are required for cell surface binding to Molt-4 cells, we asked whether the CAYHRLRRC peptide
and the peptidomimetic tat-D(KLAKLAK)₂ was also produced or at 60 min (JOURNAL OF BIOLOGICAL CHEMISTRY 11758). D(KLAKLAK)₂ was generated through Merrifield synthesis; the effect on the uptake of fam-CAYHRLRRC peptide (Fig. 6, uptake of fam-tat peptide by Molt-4 cells, whereas it had no interaction with excess amounts of heparin strongly inhibited the cells such as Molt-4 and K562 was studied (Fig. 6). Co-incubation with excess amounts of heparin, or heparinase III exposure would bind and enter the cells through the tat receptor. Next, the effect of excess amounts of heparin, or heparinase III exposure to the uptake of CAYHRLRRC or tat peptide into leukemia cells such as Molt-4 and K562 was studied (Fig. 6). Co-incubation with excess amounts of heparin strongly inhibited the uptake of fam-tat peptide by Molt-4 cells, whereas it had no effect on the uptake of fam-CAYHRLRRC peptide (Fig. 6, A versus B). Pretreatment with heparinase III markedly suppressed the internalization of tat, but it did not affect the uptake of fam-CAYHRLRRC peptide (Fig. 6, A versus B). Trypsin inhibited the uptake of both peptides to a similar degree. We also obtained similar results in experiments with K562 cells (Fig. 6, C versus D). These data indicate that the mechanism of cell internalization for the CAYHRLRRC peptide is functionally distinct from tat.

Selective Targeting of a Proapoptotic Peptide to Leukemia Cells—A previously reported proapoptotic domain, the peptidomimetic D(KLAKLAK)₂ selectively disrupts negatively charged mitochondrial membranes, but does not interact with eukaryotic cell membranes, thus, subsequently induces cell death after targeted internalization (34, 35). For evaluation of the capacity of the CAYHRLRRC peptide, a peptidomimetic drug consisting of CAYHRLRRC linked via Gly-Gly to D(KLAKLAK)₂ was generated through Merrifield synthesis; and the peptidomimetic tat-D(KLAKLAK)₂ was also produced for these experiments.

DISCUSSION

The targeted peptidomimetic CAYHRLRRC-D(KLAKLAK)₂ induced marked cell death in Molt-4 cells (Fig. 7, A and B); cell death induction was concentration-dependent. The IC₅₀ value for CAYHRLRRC-D(KLAKLAK)₂ was ~30 µM, almost identical to that of the synthetic CAYHRLRRC peptide for phage binding (Fig. 2A). The non-conjugated peptide CAYHRLRRC and the non-conjugated peptidomimetic D(KLAKLAK)₂ revealed no detectable toxic effects on Molt-4 cells at equimolar doses (Fig. 7, A and B).

The effect of CAYHRLRRC-D(KLAKLAK)₂ on the viability of a variety of tumor cells was determined and compared with that of tat-D(KLAKLAK)₂. CAYHRLRRC-D(KLAKLAK)₂ induced programmed cell death in all the leukemia/lymphoma cell lines evaluated (CCRF-CEM, K562, RPMI-8226, and U937; Fig. 7C), whereas no significant effect over the same concentration range was observed in non-leukemia cells (A549, MCF-7, and HepG2; Fig. 7D). In contrast, D(KLAKLAK)₂ peptidomimetic linked to tat induced programmed cell death of all the cell lines, data indicating that it has no particular cell selectivity. The proapoptotic chimera peptidomimetic targeted by the CAYHRLRRC peptide was therefore internalized upon binding, with high selectivity for leukemia- and lymphoma-derived cells.
mechanisms (15–20). In fact, macropinocytosis has recently been proposed as a major pathway for the endocytic uptake of arginine-rich peptides and cognate protein conjugates, although the relative contribution of alternative cell entry mechanisms such as clathrin-dependent endocytosis or energy-independent, direct membrane translocation is still not entirely clear (12–20). Be that as it may, macropinocytosis is clearly different from other pinocytosis pathways, such as clathrin-mediated, caveola-dependent, and clathrin- and caveola-independent endocytosis (18–20). That cholesterol depletion (42) and the phosphoinositide 3-kinase inhibitor wortmannin (43) inhibit membrane ruffling and macropinocytosis confirms that lipid rafts are involved in macropinosome formation (19).

Amiloride and its analogs, which inhibit the plasma membrane Na⁺/H⁺ exchange protein, also suppress this process (44). Macropinosomes are relatively large (>1 μm), and inherently leaky vesicles, in comparison to other types of endosomes (20, 29, 45). Because it is thought to be an efficient route of intracellular delivery, macropinocytosis provides potential advantages over other modes of translocation, such as an increased uptake of macromolecules, a low level of lysosomal degradation, and a rapid release of molecules to the cytosols (12, 46). Notably, membrane-associated proteoglycans (such as certain HSPGs) have recently been reported as receptors for macropinocytic uptake of arginine-rich peptides (12, 14, 47) such as HIV-1 tat (residues 48–60) and octa-arginine (Arg₈) motifs (30, 31). The mechanism underlying the translocation of cell-penetrating peptides across the lipid bilayer of mammalian cells is not as yet fully understood (29, 40); however, among commonly used cell-penetrating peptide motifs (30, 31), tat contains six Arg residues and a sequence of three Lys residues within its 13 residues, and the Arg residues play a critical role in its internalization (29, 31). Because HSPGs are widely expressed by most cells (41, 48), arginine-rich cell-penetrating peptides are promising candidate tools for targeted delivery of a wide range of molecules and drugs, which include proteins, antisense DNA, liposomes, and small interfering RNA (29, 45, 49, 50).

To select human leukemia cell-specific peptide ligands, we used the BRASIL cell-panning method, which allows selection of phage that display specific receptor-binding ligands with both high and low affinity in living cells (22). We isolated a phage displaying the CAYHRLRRC sequence selected from combinatorial biopanning on the human T-lymphoblastic leukemia Molt-4 cells, after systematic analysis of peptides binding to the NCI-60 cell lines (23). Unexpectedly, the previously selected lymph node-homing motif Cys-Ala-Tyr (32) was also part of the primary sequence within CAYHRLRRC.
The CAYHRLRRC phage and the corresponding synthetic peptide bind selectively to all leukemia and lymphoma cells that were tested, with an affinity of $\approx 30 \mu M$, prior to entry into the cells. The BRASIL method has previously contributed to the discovery of new peptide ligands that follow endocytic internalization, e.g. the GRP78-targeted peptide ligand internalized by clathrin-mediated endocytosis (51). To our knowledge, of the many peptide ligands that have been isolated by combinatorial selection of phage-display libraries, the peptide CAYHRLRRC is the first example to be internalized by macropinocytosis. The macropinocytic pathway through which CAYHRLRRC was internalized was identified by two main lines of experimental evidence: (i) the marked inhibition of fluorescent-labeled CAYHRLRRC peptide uptake by the lipid raft inhibitor MβCD and the macropinocytosis-specific inhibitors 5-(N-ethyl-N-isopropyl)amiloride and wortmannin, and (ii) the colocalization of CAYHRLRRC peptide with the specific marker ND70 (Fig. 5C). By using living cells for the detection of fluorescent-labeled peptides in cells by confocal microscopy, we avoided the artificial intracellular distribution induced by fixation (26). Macropinocytosis is one type of endocytosis that takes place in many cell types, such as dendritic cells, macrophages, fibroblasts, and epithelial cells (18–20). This process accompanies the membrane ruffling elicited by growth factors or other signals (52, 53); yet, little is known about macropinocytosis regulation in lymphoid cells (54) although it appears that the tat peptide is also internalized into Nama-Lw lymphoma cells by macropinocytosis (14). In this study, we observed significant induction of macropinocytosis by the CAYHRLRRC peptide in human T-lymphoblastic Molt-4 cells and chronic myelogenous K562 cells. These indicate that macropinocytosis may be an appropriate pathway for targeted delivery of drugs and genes into lymphoid cells. Whereas the relative contribution of the Cys-Ala-Tyr motif for the observed selectivity \textit{in vitro} remains an open question to be addressed in future studies, a reasonable working hypothesis is that the selectivity for leukemia/lymphoma cells is likely mediated by it (32).

Macropinocytosis has recently received attention as an entry route for drugs and other compounds, because its relatively leaky nature and the uptake of macromolecules (>1 \mu m) are considered to be advantageous. In clathrin-mediated endocytosis, the internalized drugs and genes attached to ligands are preferentially localized in lysosomes and therefore are readily degraded by lysosomal proteases. Poor intracellular trafficking by clathrin-mediated endocytosis is believed to limit the transfection of genes (45). Therefore, avoidance of lysosomal degradation and the ease of escape from macropinosomes (29) that characterize macropinocytosis are additional advantages for delivery systems. Some cell-penetrating peptides such as the arginine-rich peptide tat and Arg8 peptides are used as carriers to deliver various macromolecules, including proteins, peptides, oligonucleotides, plasmids, and imaging agents, because they are mainly endocytosed by a lipid raft-dependent macropinocytosis pathway (12–14), tat is also internalized via clathrin-mediated endocytosis (15, 16). We demonstrate here that the CAYHRLRRC peptide delivered the proapoptotic peptidomimetic into leukemia cells, and with higher selectivity than observed with the tat conjugate. One possible interpretation is that the CAYHRLRRC peptide may behave as a dual motif peptide with a tat-like cell-penetrating motif along with a lymph node-homing motif that bestows leukemia/lymphoma cell selectivity. In general, macropinocytosis is a receptor-independent endocytosis characterized by plasma membrane ruffling (18–20). However, in another possible interpretation, our
results are also consistent with the possibility of a specific receptor for the CAYHRLRRC peptide that triggers macropinocytosis in Molt-4 cells. Whereas it is established that HSPGs serve as receptors for the cellular uptake of tat and R8 peptides (12, 14, 15, 41, 47), the recognition specificity of HSPGs is broad, and heparan sulfate interacts with various extracellular signaling molecules, e.g. growth factors, morphogens, enzymes, and chemokines. HSPGs are expressed by both normal and cancer cells (41, 48). We observed that the uptake of CAYHRLRRC by both leukemia cell lines (Molt-4 and K562) was not affected by the presence of heparin or heparinase, whereas that of tat was completely inhibited by these molecules. Furthermore, an assay with y3(KLAKLAK)2 conjugates for cell death revealed a different specificity of peptide uptake between the CAYHRLRRC and tat peptides. From these results, one might conclude that the receptor for CAYHRLRRC is unlikely to be an HSPG with expression limited to leukemia and lymphoma cells. Epidermal growth factor and platelet-derived growth factor can transiently induce membrane ruffling that accompanies macropinocytosis in most cells (52, 53); the CAYHRLRRC peptide might act as a growth factor mimic in part by binding to cognate growth factor receptors. However, we found no sequence similarity between the peptide sequence and any known growth factors. Moreover, our attempts to identify the CAYHRLRRC receptor by peptide affinity chromatography yielded no viable candidates, this reinforcing our original experimental working hypothesis.

CAYHRLRRC-y3(KLAKLAK)2 selectively killed all the leukemia and lymphoma cells tested (Fig. 7, B–D). The cell selectivity was consonant with the relative binding of phage to these cell lines (Fig. 1). This observation indicates that CAYHRLRRC binds as a function of target abundance on the cell surface; the sequelae of this interaction are the intracellular delivery and subsequent mitochondrial membrane targeting of the proapoptotic domain y3(KLAKLAK)2. Our group and others have previously reported successful delivery of cytotoxic drugs (55), proapoptotic and cytotoxic peptides (25, 33, 35, 56), metalloproteinase inhibitors (57), cytokines (58), imaging agents (59, 60), and genes (60, 61) in tumor cells or in transgenic and gene-targeted virus (AAV) and phage (termed AAVP; Ref. 60) or genes with a hybrid viral vector consisting of targeted adeno-associated virus (AAV) and phage (termed AAVP; Ref. 60) or through AAV libraries (61). Ultimately, targeted versions of AAV (61) or AAVP (60) vectors displaying the CAYHRLRRC peptide may enable selective ligand-directed gene transfer into leukemia cells.

In conclusion, by using the cell-based phage-display technology, we found and validated a new targeting peptide, CAYHRLRRC, with cell-penetrating activity via macropinocytosis, and selectivity for leukemia/lymphoma cells. These results may have translational relevance for the development of new ligand-directed agents against human leukemias and lymphomas.

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