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Enhancement of Retroviral Gene Transduction on a Dish Coated with a Cocktail of Two Different Polypeptides: One Exhibiting Binding Activity toward Target Cells, and the Other toward Retroviral Vectors

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CH-296, a recombinant fragment of human fibronectin (FN) composed of the cell-binding domain (C-domain), heparin-binding domain II (H-domain), and CS1 site, enhances the retrovirus-mediated gene transduction (GT) of hematopoietic stem cells. The RGD sequence in the C-domain is recognized by a variety of cell types through integrin VLA-5, and the LDV sequence in the CS1-site is recognized by integrin VLA-4. Retrovirus particles were also found to bind to the H-domain. Consequently, the CH-296 fragment can enhance GT through binding to both retrovirus particles and target cells that express integrins VLA-5 and/or VLA-4. In this study, we found that the GT efficiency can be maintained at levels comparable to that of CH-271, a FN fragment similar to CH-296 but lacking the CS1 site, when a cocktail of separated functional domains of CH-271 is used. When a dish was coated with a mixture of the C-domain and H-domain (molar ratio, 1:10), the GT efficiency of NIH3T3 cells reached the same level as that of the mother fragment, CH-271. The H-domain in the cocktail can be replaced with other virus-binding components, polylysine, FGF, and the insulin-binding domain of ColV, without the loss of GT efficiency. With other than FN fragments, a cocktail of erythropoietin and polylysine caused higher GT efficiency of Epo-receptor expressing TF-1 cells than in the case of each component alone.

Key words: cell targeting, cocktail effect, fibronectin, gene transduction, retrovirus vector.

Stem-cell gene therapy is ideal for treating inherited blood disorders and for preventing side effects on blood cells caused by cancer chemotherapy (*1*). With the supernatant infection method, in which stem cells are cultured with retroviral vectors free from vector-producing cells, GT is so poor that stem cells and virus-producing cells are co-cultured to yield a reasonably high GT efficiency suitable for clinical applications (*2*). The co-cultivation method, however, cannot be applied when treating human patients due to the difficulty in complete removal of virus producer cells from the transfected target cells.

FN, a multi-functional extracellular matrix molecule, has been shown to influence the adhesion, migration, growth, and differentiation of many cell types, including hematopoietic cells (*3*).

There are three major cell binding regions of the FN molecule that serve as ligands for distinct cell surface receptors (*4*). Adhesion to the Arg-Gly-Asp-Ser (RGDS) sequence in the cell-binding domain of the FN molecule is mediated by the $\alpha_5\beta_1$ very late antigen-5 (VLA-5) integrin receptor (*5-7*). The alternatively-spliced type III connecting segment (IIICS) region of FN contains an important cell-binding site, the CS1 sequence containing LDV (*8*). Adhesion to the LDV sequence is mediated by the $\alpha_4\beta_1$ (VLA-4) integrin. In addition, carboxy-terminal heparin binding domain II (H-domain) promotes cell binding mediated by cell surface proteoglycans (*9, 10*).

Hanenberg *et al.* (*11*) found that highly efficient GT of murine hematopoietic stem cells can be achieved by culturing with producer-cell-free viral vectors on a dish coated with recombinant FN fragments produced by Kimizuka *et al.* (*12*).

Comparison of the GT efficiency with the six different FN fragments generated with the combination of three functional domains, *i.e.* the cell-binding domain (C-domain), heparin-binding domain (H-domain), and CS1 sequence (Fig. 1), has shown that CH-296, containing all three functional domains (C-domain, H-domain, and CS1 site), most efficiently enhances the retroviral GT of mammalian cells, especially hematopoietic stem cells. This result led to the working hypothesis that target cells, which express integrin receptors VLA-4 and/or VLA-5, are localized on CH-296 fragments mainly through the interaction of FN ligands, LVD in the CS1 site and/or RGD in the C-domain (*11*). Virus particles can also be bound to the CH-296 fragment through the interaction of its H-domain, composed of type III repeats III_{12}, III_{13}, and III_{14}, and some unidentified molecules (probably proteoglycans) on the surface of virus particles.

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Abbreviations: C-domain, cell-binding domain of FN; ColV, insulin- and heparin-binding domain of human type V collagen; CS1, connecting segment 1 of FN; Epo, erythropoietin; FGF, fibroblast growth factor; FN, fibronectin; GST, glutathione-S-transferase; GT, gene transduction; H-domain, heparin-binding domain II of FN; VLA4, very late antigen 4; VLA5, very late antigen 5.

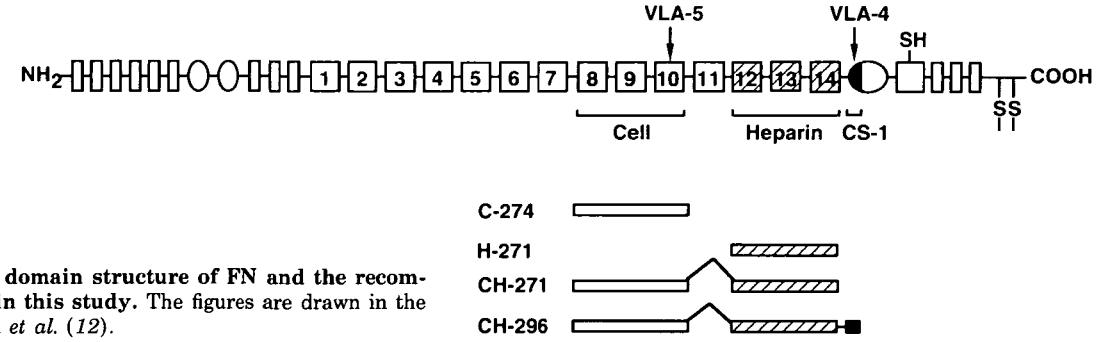

Fig. 1. **Diagram of the domain structure of FN and the recombinant fragments used in this study.** The figures are drawn in the same way as by Kimizuka *et al.* (*12*).

This hypothesis suggests that CH-296, a multi-functional FN fragment, provides the ideal "field" for the reaction between the target cells and viral vectors. However, taking into consideration the sizes of these three components, namely, target cells (20 μm, on microscopic observation), retroviral particles (80-100 nm), and FN fragment CH-296 [at the longest, 25 nm, calculated from the size of domain III_{10} (*13*)], one can easily conclude that one CH-296 molecule coated on a culture dish is too small to accommodate the other two components, namely, the target cell and retroviral particle, at the same time. The geometrical data on target cells and retrovirus particles indicate that the target cell and retrovirus particle are at least 800 times and 3 times larger than the CH-296 fragment, respectively, and suggest that the CH-296 fragment immobilized on a dish cannot bind to both the target cell and the retrovirus particle simultaneously. Rather, it is likely that one CH-296 molecule binds only to one target cell or one retrovirus particle.

If this interpretation is correct, the enhancing effect on GT of fused FN fragments should be observed without covalent connection of functional domains, each responsible for cell-binding and virus-binding.

We report here that a cocktail of separated functional domains of FN could enhance the GT efficiency of NIH3T3 cells, which express integrin VLA-5, to the same level as in the case of the mother FN fragment (CH-271), which contains cell-binding (C-domain) and retrovirus-binding (H-domain) domains. In addition, enhancement of GT of TF-1 cells, which express Epo-receptor and integrin VLA-5, was demonstrated using a cocktail of erythropoietin and polylysine instead of FN fragments.

MATERIALS AND METHODS

Retroviral Vectors and Cell Lines—The PM5neo vector packaged in the ecotropic packaging cell line, GP+E86 (*14*), the N_2/ZipTKNEO vector (*15*) packaged in the amphotropic packaging cell line, GP+envAm-12 (*16*), and murine fibroblast cell line NIH3T3 were kindly supplied by David A. Williams (Indiana University). The human hematopoietic cell lines, TF-1 and HL-60, were purchased from ATCC. HL-60 cells were cultured in RPMI 1640 (Gibco), TF-1 cells in RPMI 1640 containing 5 ng/ml of GM-CFS (Petro Tech), and all other cell lines in DMEM (JRH Bioscience). All media contained 10% fetal calf serum (Gibco) with 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco). Each virus-containing supernatant (SN) was harvested after overnight incubation, filtered through a 0.45 μm filter (Millipore), and stored at −80°C.

Preparation of Peptides—FGF (basic fibroblast growth factor; Becton Deckinson) and polylysine (poly-L-lysine hydrobromide, M_r 50,000-100,000) were purchased from Wako Pure Chemical. FN fragments C-274, H-271, C-CS1, CH-271, and CH-296 were prepared as described previously (*12*). CH-296 is commercially available from Takara Shuzo as RetroNectin™. C-FGF, a fusion protein comprising the cell-binding domain of FN and FGF, was prepared as described previously (*17*). C-FGF-CS1, a fusion protein comprising the cell-binding domain of FN, FGF and the CS1 sequence of FN, was prepared in a manner similar to that for C-FGF. ColV, the insulin- and heparin-binding domain of α1 chain of human type V collagen, and C-ColV, a fusion protein comprising the cell-binding domain of fibronectin and ColV, were prepared as described previously (*18*). GST-Epo, a fusion protein comprising glutathione-S-transferase (GST) and erythropoietin (Epo), was prepared based on the study of Bill *et al.* (*19*). Briefly, the erythropoietin gene amplified by PCR was inserted into a plasmid vector, pGEX5X-3 (Pharmacia), downstream of the coding sequence of GST in frame. GST-Epo was purified on glutathione-Sepharose 4B (Pharmacia), Resource Q (Pharmacia), and Superdex 200 (Pharmacia) columns from an extract of the relevant recombinant *Escherichia coli*.

Genetic Transduction—The efficiency of transduction was assessed principally in the same way as in the previous study with small modifications (*11*). A PBS solution of the peptides described above was added to non-tissue culture-treated dishes (35 mm; Falcon) for coating for 2 h. After the incubation, the dishes were blocked with 2% BSA (Boeringer Mannheim), and then washed with Hank's Balanced Salt Solution (Gibco) supplemented with 2.5% 1 M Hepes (Gibco). Two ml of an appropriately diluted virus supernatant (10^2-10^4 pfu/ml) was added, followed by incubation for 30 min and then thorough washing with PBS. This washing step was included to monitor the occurrence of transduction attributable only to the virus particles bound to fragments coated on a dish. This facilitates proper evaluation of the contribution to the gene transduction of each fragment, especially when adherent cells including NIH3T3 are targeted. Two thousand NIH3T3 cells were added for GT in the above dishes and incubated at 37°C for 2 h. The cells were harvested with trypsin (Gibco), and re-plated on tissue culture-treated dishes (100 mm, Iwaki) with 10 ml of medium that either did or did not contain 0.8 mg/ml G418 (Gibco). After 10-14 days, the number of G418 resistant ($G418^r$) colonies and that of total colonies

(G418 free dishes) were determined, and then the GT efficiencies, *i.e.* the ratios of the number of G418^r colonies relative to that of total colonies in % (GT%), were calculated for various peptides used to coat dishes. When TF-1 or HL-60 were the target cells, GT was examined in the same way as for NIH3T3 with the following three changes. Firstly, the washing step for removing non-bound viruses was omitted. Secondly, the number of target cells used was 10,000 instead of 2,000, and thirdly, the transduced cells were re-plated on dishes coated with CH-296. This allows colony formation by TF-1 or HL-60 cells, which otherwise cannot form colonies due to their non-adherent characteristics, for the purpose of assessing transduction efficiencies.

RESULTS

GT on a Dish Coated with a Cocktail of Two Different Functional Domains of FN

Since only one integrin receptor, VLA-5, is expressed on NIH3T3 cells, these cells are suitable for examining the cocktail effect of a CH-271 fragment, which is composed of the C-domain (C-274), ligand for VLA-5, and H-domain (H-271), exhibiting affinity to virus particles.

When a cocktail of the C-domain (C-274) and H-domain (H-271) in an equimolar ratio was coated on a culture dish, the GT efficiency of NIH3T3 cells was much lower than that with mother fragment CH-271, and even lower than that with the H-domain alone (Fig. 2a). This is consistent with the previously reported data, although different molar concentrations of each component in the cocktail were used in this study (1.7 μM) and in the previous one (0.4 μM) (*11*).

When the concentration of the C-domain was reduced to make the molar ratio of the C-domain (C-274) and the

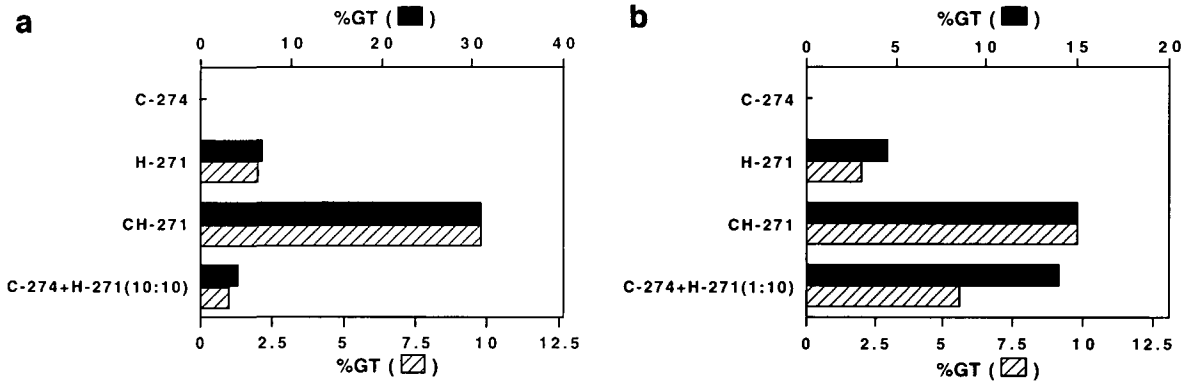

Fig. 2. **Enhancement of GT efficiency by a cocktail of fibronectin fragments.** NIH3T3 cells were used as target cells. a: Dishes were coated with 50 μg/ml of C-274 (1.7 μM), 50 μg/ml of H-271 (1.7 μM), 100 μg/ml of CH-271 (1.7 μM), and a cocktail of 50 μg/ml of C-274 and 50 μg/ml of H-271 (each 1.7 μM). b: Dishes were coated with 50 μg/ml of C-274 (1.7 μM), 50 μg/ml of H-271 (1.7 μM), 100 μg/ml of CH-271 (1.7 μM), and a cocktail of 5 μg/ml of C-274 (0.17 μM) and 50 μg/ml of H-271 (1.7 μM). The data for two independent experiments (closed columns: experiment 1, hatched columns: experiment 2) are shown.

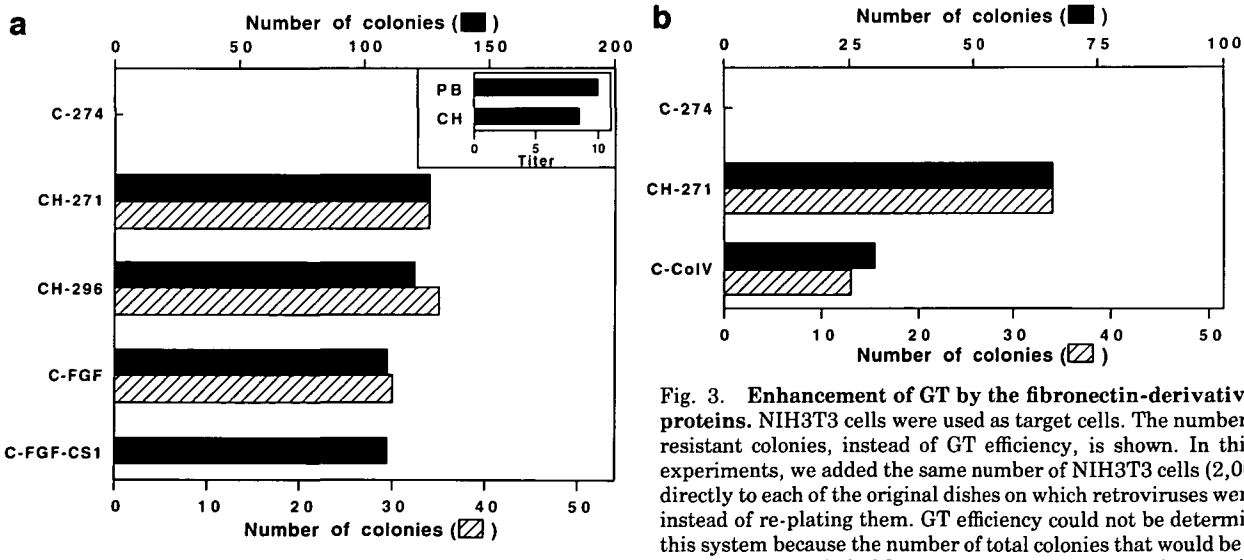

Fig. 3. **Enhancement of GT by the fibronectin-derivative fusion proteins.** NIH3T3 cells were used as target cells. The number of G418 resistant colonies, instead of GT efficiency, is shown. In this line of experiments, we added the same number of NIH3T3 cells (2,000 each), directly to each of the original dishes on which retroviruses were bound, instead of re-plating them. GT efficiency could not be determined with this system because the number of total colonies that would be obtained without the addition of G418 was too high to determine. a: Dishes were coated with 20 μg/ml of C-274 (0.7 μM), 40 μg/ml of CH-271 (0.7 μM), 42 μg/ml of CH-296 (0.7 μM), 31.5 μg/ml of C-FGF (0.7 μM), and 33.5 μg/ml of C-FGF-CS1 (0.7 μM). b: Dishes were coated with 20 μg/ml of C-274 (0.7 μM), 40 μg/ml of CH-271 (0.7 μM), and 32 μg/ml of C-ColV (0.6 μM). The data for two independent experiments (closed columns: experiment 1, hatched columns: experiment 2) are shown. The inset in (a) shows comparison of the virus titers ($\times 10^3$ CFU/ml) determined by two different methods, *i.e.*, CH-296-assisted gene transfer (CH) and polybrene-assisted gene transfer (PB).

H-domain (H-271) 1 to 10, the GT efficiency was much higher than that with each of the components alone, and reached the level in the case of mother fragment CH-271 (Fig. 2b).

We call this synergetic effect on GT efficiency of two components the "cocktail effect."

Replacement of the H-Domain in Chimeric FN Fragments by Non-FN Virus-Binding Domains—To determine whether or not the H-domain, which exhibits virus-binding activity, can be replaced with other virus-binding polypeptides, we examined the GT efficiency of two chimeric proteins, C-FGF and C-ColV, generated by Hashi *et al.* (*16*) and Hatai *et al.* (*17*), respectively, using NIH3T3 cells as target cells.

Both recombinant proteins have the C-domain of human FN tandemly connected to its carboxy-terminus with human fibroblast growth factor (FGF) (*20*) and the insulin-binding domain of collagen type V (ColV) (*21*). Another chimeric protein, C-FGF-CS1, which includes the CS1 sequence of human FN at the C-terminus of C-FGF, was also constructed.

In this study, GT assays were performed on culture dishes coated with the test chimeric proteins by first addition of virus particles, followed by extensive washing of the dishes to remove the unbound viruses, and then addition of the target cells. Therefore, the GT assay, in turn, can be used as a method of finding out the virus-binding components.

In the experiment shown in Fig. 3a, C-FGF caused levels of GT similar to in the case of CH-271, suggesting that FGF has a level of virus-binding activity similar to that of the H-domain of FN. The virus-binding activity of FGF was also confirmed in the case of the C-FGF-CS1 protein, in which the H-domain in CH-296 is replaced by FGF.

Another chimeric protein, C-ColV, in which the H-domain in CH-271 is replaced by the insulin-binding domain of ColV, was found to give 50% of the GT in the case of CH-271 (Fig. 3b).

"Cocktail Effect" of GT with a Cocktail of the C-Domain of FN and Other Virus-Binding Peptides—The above results shown in Fig. 3 clearly indicate that FGF and ColV can bind to retroviral particles. Therefore, the cocktail effect on GT with the C-domain of FN and other virus-binding peptides, FGF and ColV, was examined.

In the case of C-FGF, the "cocktail effect" was clearly observed when the culture dishes were coated with a cocktail of the C-domain of FN and FGF (Fig. 4a). The "cocktail effect," however, was not observed when an equimolar cocktail of the C-domain and ColV was used, compared with C-ColV. When the amount of the C-domain was reduced, and the molar ratio of ColV and the C-domain was changed to 10 to 3 and 10 to 1, the "cocktail effect" clearly appeared as in the case of the cocktail consisting of the C-domain and H-domain (Fig. 4b). Possible explanations for these phenomena are offered below under "DISCUSSION."

"Cocktail Effect" with TF-1 Cells as Target Cells—Although TF-1 cells are a non-adhesive cells, these cells, bearing a functional integrin receptor, VLA-5 (data not shown), can be bound on dishes coated with the C-domain of FN, as in the case of NIH3T3 cells. This time, polylysine was used as a retrovirus-binding domain instead of the H-domain of FN, in order to explore the new virus-binding components, taking into consideration its positive charges. Significant GT of TF-1 cells was observed on a dish coated with polylysine alone, indicating that both TF-1 cells and virus vectors can be localized on polylysine. When a cocktail of polylysine and C-CS1 (molar ratio, 4:1) was used, about 80% GT of TF-1 cells was attained (Fig. 4c).

The attainment of enhanced GT with four different

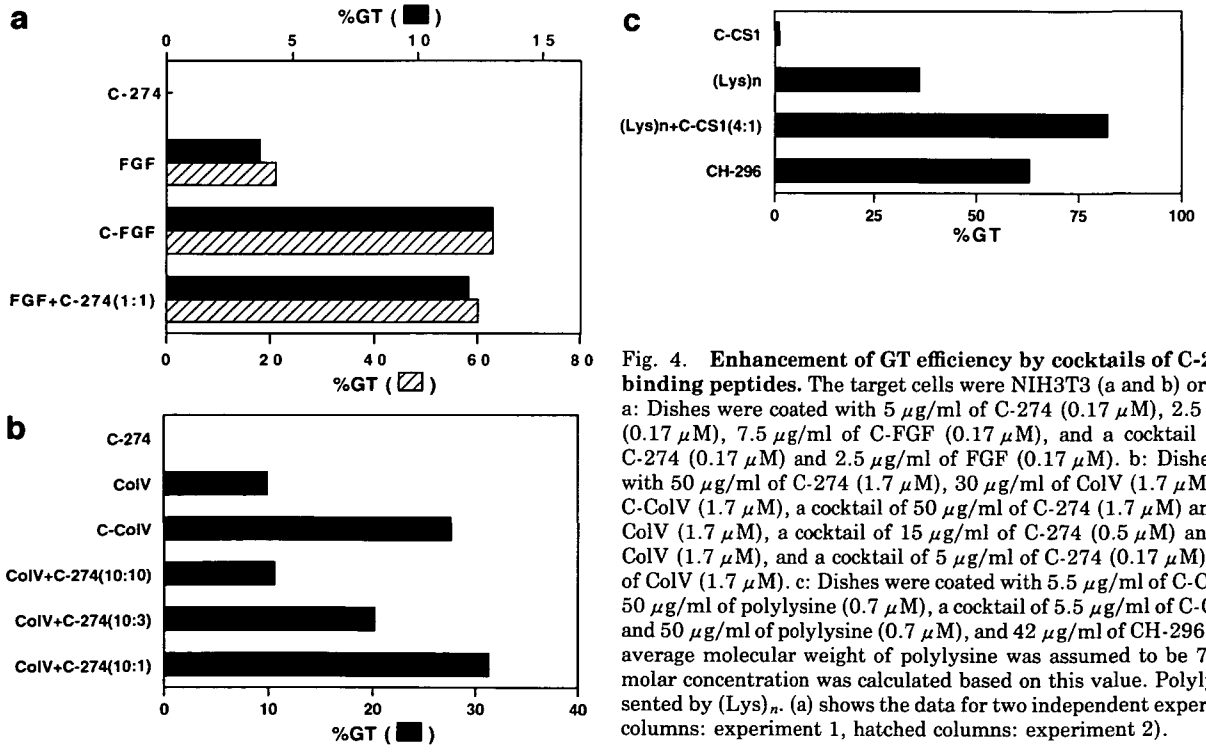

Fig. 4. **Enhancement of GT efficiency by cocktails of C-274 and virus binding peptides.** The target cells were NIH3T3 (a and b) or TF-1 (c) cells. a: Dishes were coated with 5 μg/ml of C-274 (0.17 μM), 2.5 μg/ml of FGF (0.17 μM), 7.5 μg/ml of C-FGF (0.17 μM), and a cocktail of 5 μg/ml of C-274 (0.17 μM) and 2.5 μg/ml of FGF (0.17 μM). b: Dishes were coated with 50 μg/ml of C-274 (1.7 μM), 30 μg/ml of ColV (1.7 μM), 80 μg/ml of C-ColV (1.7 μM), a cocktail of 50 μg/ml of C-274 (1.7 μM) and 30 μg/ml of ColV (1.7 μM), a cocktail of 15 μg/ml of C-274 (0.5 μM) and 30 μg/ml of ColV (1.7 μM), and a cocktail of 5 μg/ml of C-274 (0.17 μM) and 30 μg/ml of ColV (1.7 μM). c: Dishes were coated with 5.5 μg/ml of C-CS1 (0.17 μM), 50 μg/ml of polylysine (0.7 μM), a cocktail of 5.5 μg/ml of C-CS1 (0.17 μM) and 50 μg/ml of polylysine (0.7 μM), and 42 μg/ml of CH-296 (0.7 μM). The average molecular weight of polylysine was assumed to be 75,000 and the molar concentration was calculated based on this value. Polylysine is represented by $(Lys)_n$. (a) shows the data for two independent experiments (closed columns: experiment 1, hatched columns: experiment 2).

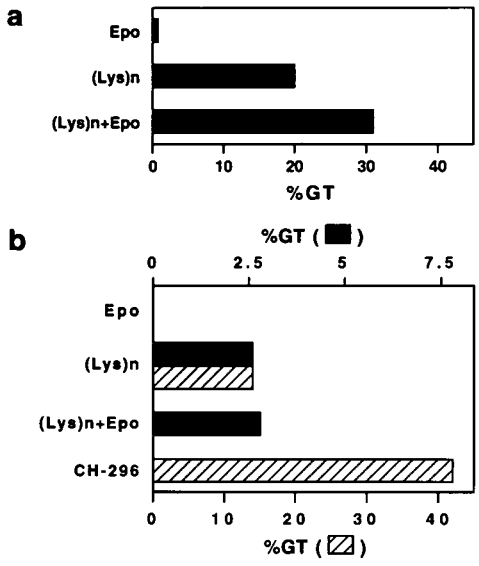

Fig. 5. **Cell-targeted GT with erythropoietin.** The targeted cells were TF-1 (a) or HL-60 (b) cells. Dishes were coated with 7.5 μg/ml of GST-Epo (0.17 μM), 50 μg/ml of polylysine (0.7 μM), a cocktail of 7.5 μg/ml of GST-Epo (0.17 μM) and 50 μg/ml of polylysine (0.7 μM), and 42 μg/ml of CH-296 (0.7 μM). The transduction data for CH-296 are only shown in (b). GST-Epo and polylysine are represented by Epo and $(Lys)_n$, respectively. In (b), the data for the first transduction experiment are shown by closed columns and those for the second transduction experiment by hatched columns.

cocktails prompted us to examine cell-specific GT with the cocktail, selecting appropriate ligands other than ligands of FN for particular target cells. TF-1 cells, which express functional Epo receptors on their surface (*22*), were chosen as target cells, with HL-60 cells that bear no functional Epo receptors as a negative control. GST-Epo, a fusion protein composed of glutathione-S-transferase and human Epo produced in *E. coli*, was used as a ligand for the Epo receptor on TF-1 cells instead of Epo. Polylysine was chosen as the virus-binding component.

On targeting TF-1 cells, the cocktail of polylysine and GST-Epo (molar ratio, 4:1) caused a significant increase in GT of the TF-1 cells that was greater than with polylysine alone (Fig. 5a). Unlike TF-1 cells, no significant cocktail effect was observed when HL-60 cells were used as the target cells (Fig. 5b). On the other hand, a significant increase in GT was observed with CH-296 even if HL-60 cells were used as target cells. These results demonstrated that cell-specific GT transduction can occur with a cocktail of any two polypeptides that exhibit binding activity toward the target cells and toward the retroviral vector instead of FN ligands.

DISCUSSION

In the previous paper, we reported that the GT efficiency of NIH3T3 with an equimolar cocktail of the C-domain (C-274) and H-domain (H-271) is much lower than that with the mother fragment CH-271, which is composed of the two domains covalently connected (*11*). However, this does not necessarily mean that the covalent connection of two functional domains, *i.e.* one for cell binding and the other for virus binding, is essential for the enhancing effect of the FN fragment. When the geometrical data on target cells, retrovirus particles and FN fragments are taken into consideration, we can easily understand that even bifunctional molecules such as CH-271 only bind to one target cell or one retrovirus particle. Furthermore, we expected that the enhancing effect could be observed even with a cocktail of two separated functional domains under the proper coating conditions. Several parameters can be used to change the coating conditions for two components, that include the concentration and/or proportion of each component, the incubation time and temperature, the order of addition to a dish of each component for coating, and so on. Here we chose the ratio of two components from among several parameters and prepared a series of dishes coated with different molar ratios to determine the proper condition that could have an enhancing effect on GT (cocktail effect). Consequently, we found that the GT efficiency was much higher than that with each component alone, and that it reached the level in the case of CH-271 if the ratio of the cocktail was set at 1 (C-domain) to 10 (H-domain).

Apparently, the C-domain in the cocktail containing either the H-domain or ColV as the virus-binding component did not contribute to the enhancement of GT, when the molar ratio of the two components was even (Figs. 2a and 4b). When the molar concentration of the C-domain was reduced compared to that of either the H-domain (H-271) or ColV, the enhancing effect on GT first appeared (Figs. 2b and 4b). One possible explanation for this phenomenon is that the coating affinity of the H-domain to the dish surface is weaker than that of the C-domain. As the result of competition between the two fragments for binding to the dish surface, the C-domain fragment (C-274) exists as a dominant molecule on the dish surface, and the enhancing effect on GT, which is mainly attributable to the virus-binding activity of the H-domain (H-271), disappears. Another possibility is that the C-domain and the H-domain somehow interact on the dish surface. The C-domain is negatively charged and the H-domain positively charged at neutral pH because the pIs of C-274 and H-271 are 5.3 and 9.3, respectively (*12*). This means that the two domains can interact at least through electrostatic forces in a medium, and that this interaction could affect binding of the H-domain to virus particles. Supposing that both domains in separated molecules interact with each other more freely than in a fused molecule, then the C-domain present in an equal molar concentration as to the H-domain can be inhibitory for the binding of the H-domain to a retrovirus. This inhibition may cause a net negative effect on GT in spite of the positive contribution derived from the binding of a target cell. When the molar concentration of the C-domain is considerably lower than that of the H-domain, *e.g.* 1/10 of the H-domain, its negative or inhibitory contribution to GT might be almost negligible, and the net positive effect on GT, which is attributed to the binding of a target cell, appears instead. Interaction between intradomains of FN has been reported from many laboratories (*23-26*), supporting our speculation concerning the interaction of the two domains.

This explains well why recombinant fragments of FN rather than the intact FN molecule are used for the GT system. For example, the LVD ligand in the CS1 site for integrin VLA-4 is buried in the intact FN molecule due to the hydrophobicity of the CS1 region (*27*).

The finding of retrovirus-binding activity in three different polypeptides, namely, H-271, FGF, and ColV, prompted us to search for common amino acid sequences that might be specifically involved in the binding of retroviruses. However, no such specific sequences were found, suggesting that the binding of the polypeptides to virus particles could be due mainly to the electrostatic force between the net charge of the polypeptides (positive) and virus particles (negative), instead of the specific interaction between particular amino acid sequences. To examine this possibility, we selected polylysine as a positively charged component with no specific sequence for virus binding. GT occurred on polylysine alone, and at a much higher efficiency with the cocktail, as shown in Fig. 4c. This supports the idea that a net positive charge of the components is essential for virus binding.

The cocktail effect observed in several sets of experiments strongly suggests that this effect is not a phenomenon specific to the interacting ligands in FN fragments, but a more general one. In principle, the cocktail effect might arise with any combination of polypeptides if one component can act as a ligand for the target cells expressing a functional receptor for the ligand, and if the other polypeptides can bind to retroviruses. The cocktail of the C-domain (C-274) and ColV, together with that of the C-domain and H-domain (H-271), also shows that the molar ratio of the two components is critical for the cocktail effect to arise. The appropriate molar ratio that can bring about the cocktail effect differs from cocktail to cocktail; an equimolar ratio is appropriate for the combination of the C-domain and FGF, and a molar ratio of 1 to 10 for that of the C-domain and either the H-domain or ColV. These phenomena support our speculation concerning why some limited molar ratios of two polypeptides can bring about the "cocktail effect." The manner of competition of two polypeptides for binding to a dish surface, or that of association of two polypeptides should depend on the combination of components. Accordingly, the inhibitory contribution to the GT efficiency of the C-domain may differ from combination to combination of the cocktail.

The level of enhancement of the cocktail effect may also vary depending on the selected ligand, even if the target cells are the same. For example, the C-domain chosen as a ligand showed a cocktail effect that was more drastic than that of GST-Epo on the same target cells, TF-1 cells. At the moment, the principal determinant that can bring about a difference in the cocktail effect is not clear. It is possible that the Epo receptor is expressed at levels lower than those of the C-domain receptor, VLA 5, or that the Epo receptor exhibits lower affinity than VLA5 to its ligands.

When we attempted to construct a fusion protein composed of Epo and H-domain (H-271) for cell-specific GT, we experienced difficulty in the purification of the fusion protein from the lysate of *E. coli* with an expression plasmid. In such a case, it was proven that the cocktail method is quite useful for saving time and labor necessary for generating fusion proteins. The optimum condition for GT was found merely by preparing cocktails with serial molar ratios of two polypeptides, and by checking the transduction efficiency of target cells.

The enhancement of GT observed with several kinds of cocktails in this study demonstrates the validity of our working hypothesis, *i.e.* the co-localization theory, and at the same time indicates that the cocktail method can be widely applied to the construction of a cell-specific targeting system for GT.

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