Target Cell-specific DNA Transfer Mediated by a Chimeric Multidomain Protein

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Based on the multidomain structure of the bacterial Pseudomonas exotoxin A, a recombinant fusion protein was constructed which serves as a target cell-specific carrier for the transfer of DNA via receptor-mediated endocytosis. The protein consists of three functional domains: 1) an ErbB-2-specific single chain antibody confers target cell specificity, 2) the exotoxin A translocation domain facilitates endosome escape, and 3) a DNA binding domain derived from the yeast GAL4 protein enables sequence-specific high affinity binding to DNA. Carrier proteins purified from bacterial lysates displayed both ErbB-2-specific and DNA sequence-specific binding in vitro. Complexes which formed spontaneously by the interaction of the fusion protein with a luciferase reporter gene construct carrying a GAL4-specific recognition sequence, after condensation of the DNA and compensation of excess negative charge with poly-L-lysine were able to transfect ErbB-2-expressing cells in vitro in a cell-specific manner. Transient expression of the luciferase gene driven by the SV40 early promoter was observed and correlates with the amount of carrier protein in the complex. Truncated forms of the carrier protein lacking either the cell recognition domain or the translocation domain failed to facilitate efficient DNA transfer.

Somatic gene therapy is based upon the introduction of therapeutically active genes into individual cells. A great variety of effector genes have been shown to hold promise for the treatment of inherited or acquired diseases in humans, but the lack of optimal gene delivery methods still presents a major limitation of such approaches. Viral vectors are widely used in gene therapy applications due to their high level of DNA uptake and expression. However, major disadvantages of viral vectors for gene transfer include a lack of cell type specificity with regard to their infectivity, a restriction in the size of incorporated DNA, and safety considerations. Alternative strategies for the delivery of DNA into target cells are being developed that are based upon the construction of artificial viral-like particles incorporating only activities required for efficient DNA transport and expression, but avoiding viral genomic information (1, 2).

Complexes of DNA with ligand-poly-L-lysine conjugates for cellular uptake via receptor-mediated endocytosis fulfill some of the requirements of such reconstructed pseudo-viral vectors and have been developed extensively during the last years (3, 4). By covering plasmid DNA with poly-L-lysine molecules covalently attached to different ligand moieties, multimolecular toroid structures can be obtained which are small enough (80–100 nm) to be engulfed by endosomes (5). However, these particles lack the ability to actively escape from the endosome before reaching the lysosomal compartment, which strongly decreases transfection efficiency (6, 7). Therefore adenovirus particles have been incorporated to supply an endosome escape activity (8–10). Also the addition of fusogenic peptides of viral origin to the DNA complex provides endosome escape activity in the absence of a viral genome, but is not able to fully reproduce the efficiency of the whole viral particle (11, 12).

Both approaches rely on natural mechanisms to escape from the endosome, which are triggered by a drop in the endosomal pH during the intracellular routing to the lysosome. Several naturally occurring proteins of non-viral origin also utilize this acidification as a signal to activate an endosome escape function. A variety of bacterial toxins are able to bind specifically to receptors on the target cell surface and, after internalization via receptor-mediated endocytosis, facilitate the translocation of biologically active protein domains from the endosome to the cytosol. In addition to cell binding and enzymatic domains these proteins carry an internal domain with translocation function (13). By replacing the cell recognition domain with antibodies or natural ligands, such bacterial toxins have been engineered to redirect toxicity to a restricted type of target cells (14) or, by modifying the enzymatic domain, have been used to transport heterologous protein domains into cells (15, 16). We have described previously a recombinant single chain toxin consisting of an antibody domain specific for the ErbB-2 receptor protein overexpressed in a high percentage of human tumors and the translocation and enzymatic domains of Pseudomonas exotoxin A (17, 18). This molecule is selectively cytotoxic in vitro and in vivo for human tumor cells overexpressing the ErbB-2 receptor protein.

Here we propose a novel approach for the target cell-specific transfer of DNA. Based on the multidomain structure of the bacterial Pseudomonas exotoxin A we have developed a fusion protein as a specific DNA carrier. It consists of three functional domains: 1) a scFv(FR5) single chain antibody domain binding to the ErbB-2 receptor confers target cell specificity, 2) the exotoxin A translocation domain facilitates endosome escape, and 3) a sequence-specific DNA binding domain derived from the yeast transcriptional activator GAL4 enables high affinity binding to DNA molecules. Complexes of the multidomain fusion protein with plasmid DNA carrying a reporter gene and a GAL4-specific recognition sequence, after condensation and charge neutralization with poly-L-lysine, are able to transfect...
ErbB-2-expressing tumor cells in vitro in a cell-specific manner. Our data suggest that this system might be useful for the target cell-specific delivery of genes with therapeutic potential.

**EXPERIMENTAL PROCEDURES**

Construction of Multifunctional Fusion Proteins—A plasmid for the bacterial expression of a multifunctional fusion protein containing the ErbB-2-specific single chain antibody (scFv), scFv(FRP5), domain II (translocation domain) of Pseudomonas exotoxin A (ETA), and the DNA binding domain of the yeast transcriptional activator GAL4 as well as plasmids encoding control proteins, which either lack the scFv or the ETA domains were constructed. The constructs are schematically shown in Fig. 1A. A XbaI/Mael DNA fragment encoding amino acids 252–366 of ETA from Pseudomonas aeruginosa was derived by XbaI/Xhol and subsequent Mael digestion of plasmid pWW20 (17). A double-stranded oligonucleotide adaptor with Mael I and EcoRi compatible ends, containing internal HindII and SacI restriction sites and a sequence encoding the endoplasmic reticulum retention signal KDEL, was constructed by annealing the oligonucleotides 5'-CGAAGAGCTTGAGGACCTCTCTGTCACTC-3' and 5'-GAACTGTTGCTGATACAGTCAACTG-3'. A KpnI/SacI fragment containing the sequence encoding the ompA signal peptide, the synthetic FLAG epitope, a cluster of 6 His residues, and the scFv(FRP5) was isolated. The scFv(FRP5) and the ETA-GAL4 fragments were fused into a single open reading frame by ligation with NdeI/EcoRi-digested plasmid pFLAG-ompA (IBI Biochemicals). The resulting plasmid pPSV50-SEG was used for the expression of the scFv(FRP5)-ETA-GAL4 fusion protein SEG in Escherichia coli.

The multiple cloning site of plasmid pSW50 (18) was modified by inserting a double-stranded oligonucleotide containing HindII, XbaI, KpnI, SacI, EcoRi, BglII, and Xhol restriction sites between the original HindII and EcoRi sites of pSW50. The resulting plasmid pSF50 was used for the construction of SEG-derived fusion genes lacking either the scFv(FRP5) or the ETA domain. The HindII restriction site between the ETA and the GAL4 domain in plasmid pWW35 was destroyed and used for the construction of 5EG-derived fusion genes lacking either the ETA and the GAL4 domain in plasmid pWW35. A fragment encoding the ETA-GAL4 fusion and recognition motifs (see below). Five pmol of the DNA oligonucleotide were annealed with 500 pmol of the double-stranded DNA adaptor (G4) containing two consecutives GAL4 recognition sequences in the 3'- and 5'-end of the oligonucleotide (see below). Five pmol of the DNA oligonucleotide were annealed with 500 pmol of the double-stranded DNA adaptor (G4) containing two consecutives GAL4 recognition sequences in the 3' and 5' ends of the oligonucleotide. The final construct pSV2G4LUC contains the luciferase reporter gene under the control of the CMV early promoter and the GAL4 recognition motifs (see below). Five pmol of the DNA oligonucleotide were annealed with 500 pmol of the double-stranded DNA adaptor (G4) containing two consecutives GAL4 recognition sequences in the 3' and 5' ends of the oligonucleotide.

**Construction of the Luciferase Reporter Gene Plasmid**

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was isolated from E. coli XL-1 blue (Stratagene) using a plasmid purification kit (QuiaGen) according to the manufacturer’s recommendations. For transfection, cells were seeded on 12-well tissue culture plates at a density of \(4 \times 10^5\) cells/well and grown overnight at 37°C. Growth medium was exchanged with 1 ml/well of fresh medium 5 h before the addition of protein-DNA complexes. Complexes were prepared by incubation of 4 \(\mu\)g pSV2GAL4UC plasmid with 240 \(\mu\)g of fusion protein in a buffer containing 50 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl\(_2\), 100 \(\mu\)M ZnCl\(_2\) for 15 min at room temperature. To facilitate condensation of the protein-DNA complex, 2.5 \(\mu\)g of poly-l-lysine HBr (Sigma) with an average degree of polymerization of 236 residues (pl236) were added slowly to the complex with a constant flow of air bubbles, and the mixture was incubated for an additional 15 min. The optimal amount of pl236 was determined as the quantity required for the complete retardation of plasmid DNA in a 1% agarose gel (data not shown). A pl236:DNA molar ratio of approximately 50:1 was determined which coincides with the estimated theoretical molar ratio in an electroneutral pl236:DNA complex. Under these conditions DNA was completely protected against DNase I treatment (10 \(\mu\)g/ml) for 1 h at 37°C. Only minimal DNA degradation (~10%) was observed after incubation in 10% FCS for 20 h at 37°C (data not shown).

The protein-DNA complex was added to the cells. The final concentration of the components in the complex during cell incubation was 1 \(\mu\)g DNA, 4 \(\mu\)g fusion protein, and 50 \(\mu\)g pl236. The cells were incubated at 37°C for 16 h, then the medium was exchanged, and the cells were incubated for another 36 h before they were harvested for analysis.

**Luciferase Assay**—After removal of the medium the cells were washed twice with PBS and lysed for 15 min at room temperature in 100 \(\mu\)l of buffer containing 25 mM glycyglycine, pH 7.8, 1 mM dithiothreitol, 15% glycerol, 8 mM MgSO\(_4\), 1 mM EDTA, and 1% Triton X-100. The lysates were cleared by centrifugation, and protein content was determined by the Bradford method (23). Fifty \(\mu\)g of the lysate were mixed with an identical volume of dilution buffer containing 25 mM glycyglycine, pH 7.8, 10 mM MgSO\(_4\), and 5 mM ATP. Luciferase activity was monitored for 30 s in a luminometer AutoLumat LB 953 (Berthold) with automatic injection of 300 \(\mu\)l of luciferin solution containing 250 \(\mu\)M luciferin, 25 \(\mu\)M glycylglycine, pH 7.8, and 0.5 mM coenzyme A (Boehringer Mannheim). Luciferase activity was determined as relative light units/mg of cellular protein.

**RESULTS**

Construction and Bacterial Expression of the Multifunctional DNA Carrier Protein 5EG—DNA fragments encoding the ErbB-2-specific single chain antibody domain scFv(FRP5), domain II (translocation domain) of Pseudomonas exotoxin A, and the DNA binding domain of the yeast transcriptional activator GAL4 were derived from previously described plasmids by restriction enzyme digestion or polymerase chain reaction amplification with specific oligonucleotide primers and assembled into a single open reading frame in the bacterial expression vector pSw50 (18). Plasmid pSw50-5EG encodes under the control of the yeast transcriptional activator GAL4, amino acids 2–147 of the yeast GAL4 protein (DNA binding domain), and a C-terminal KDEL endoplasmic reticulum retention signal. 5G is a similar chimeric protein lacking the translocation domain and the KDEL signal, while EG is the corresponding molecule without the cell recognition domain. 5G, SDs-polyaspartamidagel electrophoresis analysis of the 5EG protein purified from bacterial lysates. Lane 1, bacterial lysate cleared by ultracentrifugation; lane 2, eluate from a Ni\(^{2+}\)-saturated chelating Sepharose column with 250 mM imidazole; lane 3, sample 2 after concentration by ultrafiltration; lane 4, immunoblot analysis of sample 2 with a monoclonal antibody specific for the GAL4 DNA binding domain; M, molecular weight standards.

Fusion proteins were pooled, imidazole and denaturant were removed by dialysis, and the proteins were concentrated by ultrafiltration. Fig. 1B shows a SDS-polyacrylamide gel electrophoresis analysis of the 5EG fusion protein (>90% pure) after a single round of purification. The protein was detected as a single band after immunoblotting with a monoclonal antibody specific for the GAL4 DNA binding domain (Fig. 1B, lane 4). The same band appeared when a polyclonal exotoxin A antiserum (17) or a polyclonal anti-scFv antibody (25) were used for detection (data not shown).

**ErbB-2-specific Binding of 5EG**—The individual domains of the 5EG fusion protein were functionally characterized. The first domain examined was the one responsible for target cell recognition, a single chain Fv domain derived from the ErbB-2-specific monoclonal antibody FRPS (27). The recombinant 5EG protein was tested for its ability to bind to ErbB-2 in ELISA experiments. 5EG at concentrations ranging from 1 \(\text{nmol}\) to 1 \(\mu\text{mol}\) was added to the wells of 96-well plates coated with purified recombinant extracellular domain of ErbB-2, and specifically bound protein was determined. The results are shown in Fig. 2A. The apparent binding affinity of 5EG to purified ErbB-2 protein, estimated as the half-maximal saturation value, was 35 \(\text{nmol}\). Similar results were obtained in ELISA experiments using immobilized ErbB-2 overexpressing SKBR3 human breast carcinoma cells as antigen (data not shown).

**Specific DNA Binding Activity of 5EG**—The activity of the
A concentration of the 5EG fusion protein ranging from 240 pM to 1 nM. Immobilized recombinant protein comprising the extracellular domain of ErbB-2 was incubated with increasing concentrations of unlabeled specific oligonucleotide (Fig. 2B). Addition of 5EG to the GAL4-specific oligonucleotide resulted in the appearance of two bands of lower electrophoretic mobility. Since GAL4 binds to DNA as a dimer, and the sequences involved in GAL4 dimerization are present in the 5EG fusion protein (20), the bands could correspond to protein-DNA complexes containing either one or two 5EG dimers bound to the oligonucleotide. The band of the lowest electrophoretic mobility is stronger and could correspond to a high molecular weight complex containing four 5EG molecules covering most of the oligonucleotide sequence, thereby drastically reducing its capacity to migrate in the gel. The specificity of DNA binding was analyzed by competition of the complex with increasing concentrations of unlabeled specific oligonucleotide (Fig. 2, B and C). A reduction in the intensity of the shifted band was observed which clearly correlates with the ratio of unlabeled to labeled oligonucleotide.

Binding of a 5EG protein-DNA complex containing digoxigenin-labeled GAL4-specific oligonucleotide to ErbB-2 was also analyzed. In an ELISA experiment with immobilized ErbB-2 overexpressing SKBR-3 cells specifically bound complex could be detected with an anti-digoxigenin AP-coupled antibody, indicating that both binding domains of 5EG are active simultaneously (data not shown).

5EG Fusion Protein Mediates Transfer of DNA into Cells in a Dose-dependent Manner—5EG carrier protein-mediated gene transfer was analyzed using the reporter gene plasmid pSV2G4LUC described under "Experimental Procedures," which encodes the firefly luciferase gene under the control of the SV40 early promoter and contains two consecutive GAL4 recognition motifs in the 3′-untranslated region of the expression cassette. Purified 5EG fusion protein and circular pSV2G4LUC plasmid DNA were mixed at different molar ratios to allow the formation of protein-DNA complexes. Subsequently poly-L-lysine pl$_{236}$ was added to the mixture.

The final complex was added to COS-1 SV40-transformed monkey kidney cells in standard growth medium. Control cells were treated with a complex containing plasmid DNA and poly-L-lysine, but lacking the 5EG fusion protein. COS-1 cells express approximately 2 × 10$^5$ ErbB-2 molecules/cell. Monoclonal antibody FRP5 which is specific for human ErbB-2, cross-reacts with the ErbB-2 homolog of COS-1 cells (data not shown). Cells were harvested after 48 h, and the expression of the luciferase reporter gene was analyzed in cell lysates (Fig. 3). A clear linear correlation ($r = 0.991$) between the amount of protein and the level of reporter gene expression was observed, when a constant amount of plasmid DNA was used in the complex. Control cells treated with a complex lacking the 5EG carrier protein displayed only background luciferase activity.

Due to its large size and high negative charge, spontaneous uptake of DNA into cells is prevented. Both, neutralization and condensation of the DNA can be achieved by the interaction with polycationic reagents (28, 29). A strong correlation between DNA condensation and cellular uptake of ligand-DNA complexes has been shown previously (5). In the 5EG protein-DNA complex, the presence of poly-L-lysine was essential, since neither plasmid DNA alone nor 5EG protein-DNA complex in the absence of the polycation were able to successfully transfect

**Fig. 2. Functional characterization of the 5EG multidomain carrier protein.** A, the ErbB-2-specific binding of 5EG was analyzed in an ELISA experiment. Immobilized recombinant protein comprising the extracellular domain of ErbB-2 was incubated with increasing concentrations of the 5EG fusion protein ranging from 240 pM to 1 nM. Specifically bound protein was detected with a polyclonal rabbit antiserum raised against purified Pseudomonas exotoxin A followed by alkaline phosphatase-coupled goat anti-rabbit antibody and photometric detection of the conversion of the phosphatase substrate p-nitrophenyl phosphate at 405 nm. B, the binding of the 5EG protein to DNA was analyzed in a band shift assay. 1 pmol of 5EG protein was incubated with 50 fmol of 32P-labeled GAL4-specific oligonucleotide containing a tandem repeat of the GAL4 recognition motif 5′-CGAGGACAGTCTCCGG-3′ and separated on a nondenaturing polyacrylamide gel (lane 1). The positions of two bands with decreased electrophoretic mobility and the free probe are indicated by arrows. The more intense higher molecular weight complex represents only one bound dimer (complex 2:1). To show the specificity of the binding, increasing amounts of the nonradioactive probe ranging from 50 fmol to 12.8 pmol were added to the binding mixture as a competitor (lanes 2–6). C, correlation between the amount of nonradioactive competitor added and the relative amount of the radioactive probe in the higher molecular weight complexes in B. The relative radioactivity was quantified using a FUJIX BAS1000 phosphorimagier.
COS-1 cells and result in significant luciferase activity (data not shown). Also protection of the DNA from nuclease activity is important. At the poly-L-lysine pL236:DNA molar ratio of 50:1 established experimentally to achieve an electroneutral complex, no DNA degradation was observed after incubation of the complex with 10% FCS at 37 °C for 1 h. In addition, no difference in transfection efficiency was observed when COS-1 cells were incubated with SEG-DNA-pL236 complex in tissue culture medium in the presence or absence of 10% FCS (data not shown).

5EG-mediated Gene Transfer Is Target Cell-specific—The target cell specificity of 5EG-mediated gene transfer was investigated. Three different cell lines, two expressing high levels of the ErbB-2 protein on the cell surface (SKBR-3 and COS-1 cells) and one lacking the receptor (MDA-MB468 cells) were tested for their ability to express the luciferase reporter gene after transfection with the 5EG-pSV2G4LUC-pL236 complex. The cells were incubated with protein-DNA complex at a final concentration of 1 nM pSV2G4LUC plasmid DNA, 4 nM 5EG fusion protein, and 50 nM pL236. Control cells were treated with a complex lacking the 5EG carrier protein. No toxic effects were observed on the cells treated with the protein-DNA complex in comparison with untreated cells (data not shown). High levels of luciferase activity were detected 48 h after transfection in extracts of monkey COS-1 and SKBR-3 human breast carcinoma cells expressing approximately 2 × 10⁶ and 1 × 10⁴ ErbB-2 molecules per cell, respectively (Ref. 18 and data not shown) (Fig. 4A). In contrast, no luciferase expression above background levels was detected in MDA-MB468 human breast carcinoma cells which do not express detectable ErbB-2 levels (18).

Highest luciferase activity was observed in COS-1 cells, although SKBR-3 cells express approximately five times more ErbB-2 protein. This might be explained by episcopal replication and amplification of the pSV2G4LUC reporter plasmid in the SV40 T-antigen expressing COS-1 cells, facilitated by the plasmid's SV40 origin of replication (30). The level of nonspecific transfection with the pSV2G4LUC-pL236 complex lacking

![Image of graph showing luciferase activity](http://www.jbc.org/fig4.png)
required for the activity of the wild-type toxin (26). The 5G molecule displayed ErbB-2-specific binding in ELISA experiments and formed a complex with a DNA oligonucleotide containing the GAL4 recognition motif in gel retardation assays (data not shown). The ability of the 5G fusion protein to bind to ErbB-2 was also confirmed in a competition experiment. Immobilized SKBR-3 cells were incubated with the parental 5EG molecule in the presence of 5G. Specific binding of 5EG was then detected with an antibody specific for exotoxin A (Fig. 5A). Increasing concentrations of the 5G competitor led to a reduction of 5EG binding to SKBR-3 cells.

The transfection efficiency of complexes containing the truncated 5G carrier protein was analyzed in SKBR-3 cells (Fig. 5B). The cells were incubated with complexes containing pSV2G4LUC plasmid DNA, poly-L-lysine pL236, and either 5G or 5EG carrier protein. In addition, a complex containing 5EG and a 3-fold molar excess of 5G was used. Control cells received pSV2G4LUC-pL236 complex. Luciferase expression as an indicator for gene transfer activity could be detected in cells treated with 5G containing complex. However, in comparison with 5EG-mediated gene transfer the activity of 5G was reduced 6-fold. When a 3-fold molar excess of 5G protein was added to the 5EG-containing protein-DNA complex, 5EG-mediated gene transfer activity was completely abolished. Such a strong competition effect at a molar ratio of 3:1 was unexpected. It might be explained by differences in 5G and 5EG DNA binding activities and/or an inability of heterologous 5EG/5G-DNA complexes to translocate efficiently through endosomal membranes.

Endosomal acidification results in a conformational change of internalized Pseudomonas exotoxin A, a step required for the functional activation of the translocation domain and, after cleavage by a cellular protease, successful transfer of the enzymatically active C-terminal fragment into the cytosol (31). In order to characterize the activity of the exotoxin A translocation domain in the 5EG fusion protein, transfection experiments were carried out in COS-1 cells in the presence of agents which block endosomal acidification. When COS-1 cells were treated with complexes containing pSV2G4LUC plasmid DNA, poly-L-lysine pL236 and 5EG carrier protein in the presence of the acidotropic reagent chloroquine (100 μM), instead of a reduction we observed a 1.5-10-fold increase in transfection efficiency (data not shown). Similar results have been reported previously when the effect of chloroquine on gene transfer mediated by transferrin-poly-L-lysine conjugates in the presence of the fusogenic peptide Infl 1 was investigated (11). This could be explained by the dual activity of chloroquine. Chloroquine is a vacuolar amine which is freely membrane permeable in its unprotonated form, but becomes trapped inside the vesicle when it binds a proton (32). This leads to an inhibition of endosomal acidification, but due to an increase of the intravesicular concentration of the molecule, it also leads to osmotic swelling, which could result in the disruption of the endocytic vesicles and the release of internalized DNA.

Therefore in a second approach the macrolide antibiotic bafilomycin A1 was used, a potent inhibitor of vacuolar H⁺-ATPases that selectively blocks endosomal acidification (33). COS-1 cells were incubated with complexes containing pSV2G4LUC plasmid DNA, poly-L-lysine pL236 and 5EG carrier protein with or without the addition of 200 nm of bafilomycin A1. Luciferase expression as an indicator for gene transfer activity was measured. The results are shown in Fig. 5C. The presence of bafilomycin A1 resulted in a 4-fold reduction of luciferase activity showing that 5EG-mediated DNA transfer is dependent on endosomal acidification. Similar results have been reported previously when endosome-disruptive peptides

![Figure 5](http://www.jbc.org/). **Efficient gene transfer depends upon the presence of the translocation domain.** A, an ErbB-2-specific DNA-binding protein lacking the translocation domain (5G) was constructed in order to analyze the importance of this domain in the gene transfer process. ErbB-2-specific binding of 5G was analyzed in ELISA experiments. Samples containing 25 nm 5EG and increasing concentrations from 0 to 166 nm of the 5G protein as a competitor were added to formaldehyde-fixed SKBR3 cells. Specific binding of 5EG was detected with a polyclonal rabbit antiserum raised against purified Pseudomonas exotoxin A. B, SKBR3 cells were treated with 5G or 5EG containing protein-DNA complexes and the expression of the luciferase reporter gene was analyzed as described in the legend of Fig. 4. Competition between both proteins was determined by coinoculation of 5EG with a 3-fold molar excess of 5G. C, the effect of the specific inhibitor of vacuolar H⁺-ATPases bafilomycin A1 on 5EG fusion protein mediated gene transfer was analyzed in COS-1 cells. Bafilomycin A1 was added to the cells at a concentration of 200 nm 30 min before the treatment with the protein-DNA complex and maintained during the time of the experiment.
were used to enhance gene transfer (12). COS-1 cells were also 
iculated with the protein-DNA complex in the presence of 
carboxylic ionophores, another group of reagents which elevate 
vacuolar pH. Either the addition of monensin (10 \( \mu \)M) or niger-
cin (5 \( \mu \)m) to the cells decreased the transfection efficiency to 
background levels (data not shown). These data suggest that 
the activity of the translocation domain in the 5EG protein is 
very similar to its activity in the parental exotoxin A molecule.

**DISCUSSION**

Endocytosis is a major cellular mechanism for the uptake of 
macromolecules from the environment. Therefore a rational 
way to develop a system for the introduction of genetic material 
into cells in a nonaggressive manner is to take advantage of 
this natural mechanism. Receptor-mediated gene transfer has 
been successfully used to deliver reporter genes to cultured 
cells (6, 34) and to target either marker genes (35, 36) or 
therapeutical genes (37–39) to specific organs in an adult ani-
mal. Although still not as efficient as viral vectors, it has 
several advantages, which include high target cell specificity, 
lack of infection risk, no restriction in DNA size, and possibly 
lower immunogenicity. Most current approaches for receptor-
mediated gene transfer are based on a ligand moiety chemically 
conjugated to poly-L-lysine that conforms DNA binding capacity 
and facilitates the formation of condensed DNA-conjugate com-
plexes of toroid structure. In addition endosome disrupting 
activities of viral origin have been included either in trans or 
via a second poly-L-lysine conjugate in order to improve the 
transfection efficiency (3).

Here we present an alternative approach for a non-viral gene 
transfer vector that differs in several aspects from previously 
described systems. We have used molecular biology techniques 
to engineer a modular multidomain DNA carrier protein that 
combines in a single polypeptide chain all functions required 
for efficient target cell-specific gene transfer, i.e. target cell 
recognition, specific DNA binding, endosome escape, and a 
signal for nuclear transport. The fusion protein can be ex-
pressed as a recombinant molecule in bacteria and be purified 
to homogeneity. Chemical modification reactions which require 
relatively high amounts of purified material and sometimes 
result in products which are difficult to characterize can be 
avoided.

The 5EG carrier protein includes amino acids 2–147 of the 
yeast GAL4 transcriptional activator at the C terminus as a 
sequence-specific DNA binding domain. This type of domain 
was chosen because of the specificity and high affinity of its 
binding to DNA (20). The presence of a natural nuclear local-
ization signal provides an additional advantage. Other nuclear 
proteins conferring DNA binding activity and nuclear tropism 
have been used previously, but all of them were structural 
proteins (histones, protamines) with no sequence specificity 
(40–43). The introduction of specificity of DNA binding opens 
the possibility to insert the recognition motif at different non-
vertical positions in the DNA to be transferred and allows to 
predetermine the number of ligand moieties per DNA molecule 
and thereby to control the efficiency of its cellular uptake. As 
demonstrated in an electrophoretic mobility shift assay the 
5EG fusion protein spontaneously forms complexes with dou-
ble-stranded DNA carrying the GAL4 recognition motif. These 
complexes appeared to be of the expected stoichiometric com-
position, suggesting that well defined protein-DNA complexes 
could be generated simply by mixing appropriate amounts of 
DNA and carrier protein.

High affinity binding of the carrier protein to DNA appears 
desirable for two main reasons. First, the carrier protein might 
dissociate from the DNA more slowly which would extend the 
half-life of intact protein-DNA complexes. Second, after inter-
nalization by target cells it might allow to retain DNA binding 
in the endosomal environment. Endosome escape of the DNA 
still bound to the protein domain which carries a nuclear local-
ization signal could support subsequent nuclear transport of 
the DNA molecule.

The 5EG fusion protein carries a single chain Fv domain 
derived from the ErbB-2-specific monoclonal antibody FRP5 as 
a target cell recognition domain. The ErbB-2 molecule is a 
member of the EGF receptor-related family of growth factor 
receptor tyrosine kinases that play an important role in the 
development of human malignancies. In particular overexpres-
sion of ErbB-2 has been observed in a high percentage of 
tumors of epithelial origin and could be correlated with an 
unfavorable patient prognosis (44). Because of its enhanced 
expression on tumor cells, its extracellular accessibility and its 
ability to internalize after ligand binding, ErbB-2 presents a 
suitable target for directed tumor therapy. The scFv(FRP5) 
domain has been incorporated previously in fusion proteins to 
target enzymes or cytotoxic proteins to ErbB-2 expressing cells 
(17, 18, 25).

Monoclonal antibodies chemically conjugated to poly-
L-lysine have been used previously to target DNA to cells either alone 
(45) or with the help of cationic liposomes (46) or inactivated 
adenoviral particles (47). The use of a single chain antibody 
domain for cell recognition allows the construction of smaller 
DNA carriers and could reduce the immunogenicity of the final 
protein-DNA complex in vivo applications. The idea of re-
ducing the size of the ligand (“minimal ligands”) has already 
been exploited for the development of gene transfer systems, 
especially in the case of asialoglycoprotein receptor targeted 
 vectors (48–51). Recently a recombinant Fab molecule where 
the antibody heavy chain sequences were fused to protamine as 
a DNA binding domain has been used to target a suicide gene 
construct to cells expressing human immunodeficiency virus-1 
glycoprotein 120 (43).

When ligand-poly-L-lysine conjugates were used for DNA 
transfer in the absence of an endosome escape function no or 
only low expression of transferred reporter genes was usually 
observed (49, 52). The ligand-DNA complexes were able to bind 
to cells and could be internalized but most of them were trans-
ported to the lysosomal compartment where they were de-
graded by hydrolytic enzymes. The addition of acidotropic 
agents such as chloroquine or membrane destabilizing agents 
such as adenoviral particles or fusogenic peptides enhanced 
considerably the transfection efficiency (6, 8–12). Intracellular 
transport of membrane-bound constituents during endocytosis 
is pH-dependent. Most natural mechanisms employed by vi-
ruses or bacterial toxins which avoid lysosomal degradation 
take advantage of endosomal acidification to trigger a reaction 
which facilitates the exit to the cytosol.

The composition of the 5EG DNA carrier protein is based on 
the multidomain structure of the bacterial Pseudomonas exo-
toxin A. 5EG contains an internal translocation domain repre-
senting amino acids 252 to 366 of the toxin. Wild-type exotoxin 
A, after binding to target cells via its N-terminal cell binding 
domain, is internalized. In the acidic environment of the endo-
some a conformational change occurs, the toxin is proteolyti-
cally cleaved within the translocation domain between Arg-279 
and Gly-280 followed by the reduction of a disulfide bond, and 
the C-terminal fragment carrying the enzymatically active ef-
fector domain translocates to the cytosol (31). Exotoxin A has 
been used previously to facilitate the internalization and trans-
location to the cytosol of heterologous peptides or protein 
domains located C-terminal of the translocation domain (15, 16). 
The C-terminal GAL4 domain of the 5EG protein could be 
released into the cytosol via a similar mechanism. Thereby the
high affinity binding of the GAL4 domain to the DNA might allow a stable interaction. However, it was not clear whether a plasmid DNA as a second molecule covalently attached to the C-terminal domain could be released from the endosomal compartment simultaneously. Our data support the hypothesis that DNA transfer is mediated by the translocation domain, since both the use of a carrier protein lacking this domain and the blockage of endosomal acidification resulted in drastically reduced transfection efficiency.

The amino acid sequence REDLK, which is located at the very C terminus of exotoxin A and resembles the mammalian endoplasmic reticulum retention signal, has been shown to be important for toxin activity. Indeed, when the REDLK sequence was replaced by the mammalian KDEL signal, a toxin with enhanced activity was obtained, suggesting that this signal plays a role in intracellular routing (26). Therefore the KDEL signal was also included at the C terminus of the 5EG signal is absolutely required for efficient endosomal release of the GAL4-DNA complex.

Improvement of transfection efficiency by endosomolytic agents used in trans requires relative high concentrations: 50–100 μM chloroquine (11, 50, 52); 10−1011 adenoviral particles (approximately 104 viral particles/cell) (47, 53, 54), or 10–100 μM fusogenic peptides (12, 48, 49). This limits its applicability in vivo and has therefore prompted researchers to physically attach such agents directly to DNA transfer complexes (9, 10, 55) in order to increase the effectiveness of the transferrin-mediated DNA transfer to be 10–15 (5). In natural gene delivery systems such as viruses the number of cell binding moieties (spikes) per particle is several hundreds. Therefore the current protein/DNA molar ratio in our system of 4:1 might still be suboptimal and the introduction of additional protein binding sites in the DNA molecule could present one way for further improvement.

Structural domains which are able to fold independently from other regions in the parental protein are likely to retain their functionality as isolated domains and are good candidates for the use in chimeric fusion proteins. We have shown that such heterologous protein domains of mammalian, bacterial, and yeast origin can be assembled into a functional chimeric protein that thereby acquires a novel biological activity. The SEG DNA carrier protein contains in a single polypeptide chain all the activities required for the introduction of exogenous genetic material into mammalian cells and has been shown to facilitate efficient transfection of human tumor cells in a cell-specific manner. Due to the modular structure of the SEG carrier protein also similar vectors with modified activities, e.g. different target cell specificity, could be obtained by replacing individual protein domains. Such carrier proteins might become useful in gene therapy protocols for the target cell-specific delivery of genes with therapeutic potential.
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