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Optimization of Cell Surface Binding Enhances Efficiency and Specificity of Molecular Conjugate Gene Delivery*

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Molecular conjugates, or polyplexes, are promising synthetic vectors for targeted, *in vivo* gene delivery, if their efficiency can be improved. Gaining mechanistic information on conjugate gene delivery can potentially yield significant improvements in transfer efficiency by revealing barriers to conjugate transfer from the cell surface to the nucleus. We have developed an experimental system that employs epidermal growth factor as the ligand to direct delivery of DNA encoding the green fluorescent protein to mouse fibroblasts. We report here that the initial step of delivery, binding of the conjugate to the cell surface, is a barrier to gene transfer. We examined the effects of conjugate charge, ligand cross-linker spacer length, and ligand valency on polyplex cell surface binding, internalization, and gene transfer. We find that delivery is both efficient and specific only within a relatively narrow window of conjugate charge, results that correlate with binding and internalization of radiolabeled conjugate. In addition, increasing the cross-linker length can improve binding affinity and delivery. Finally, there is a significant optimum in gene delivery as a function of ligand valency, due to saturation of receptor binding and internalization. Optimizing parameters that affect surface binding therefore improves the efficiency and specificity of molecular conjugate gene delivery.

The molecular conjugate, or polyplex, is a synthetic gene delivery vector composed of nucleic acids condensed with polycations that can be cross-linked to a ligand for cell targeting. After binding to the cell surface, conjugates are internalized, and a small fraction of them escapes the endocytic network and translocates to the nucleus, where genes within the DNA of the polyplex are expressed. Investigations of conjugates have demonstrated the ability for specific receptor-mediated delivery via a wide variety of ligands (1–5), successful use of a number of cationic polymers or peptides (1, 6–9), the capacity to carry large DNA constructs (10), the potential for relatively low immunogenicity (11, 12), protection of their nucleic acid cargo from nuclease degradation (13), and gene expression for varying lengths of time after delivery, up to several months in one report (14). Despite significant promise and progress, however, conjugate delivery efficiency must be further improved before it can match that of many viral vectors.

There is strong evidence that escape from the endocytic network poses a significant obstacle to polyplex gene delivery (10, 15, 16). Mechanistic study of the cell biology of gene transfer, however, can reveal additional barriers to gene delivery, as well as identify means to engineer conjugates to overcome those barriers. One of these potential obstacles is the initial step of cellular delivery, binding of the polyplex to the cell surface. Several properties of the molecular conjugate can affect how it interacts with a cell surface. First, the cell surface membrane is negatively charged due to its lipid, glycoprotein, and proteoglycan components (17). Therefore, since the net charge of molecular conjugates may determine the degree of binding due to specific receptor-ligand *versus* nonspecific charge interactions, the overall ratio of polycation to nucleic acid in a conjugate preparation can significantly affect delivery efficiency and specificity. In addition, cross-linking a ligand to conjugates can potentially reduce its affinity for a receptor and thereby lessen conjugate binding and delivery. Finally, since a cell expresses a limited number of receptors, the number of ligands presented by each molecular conjugate can determine how rapidly they deplete the receptor pool.

We have previously described a system that employs epidermal growth factor (EGF)[1] as a ligand to direct delivery of a gene encoding GFP to mouse fibroblasts expressing the EGF receptor (18). Conjugates are generated by condensing plasmid DNA with polylysine cross-linked to streptavidin, followed by tethering biotinylated EGF to the conjugate surface. Using this system, we have investigated whether polyplex binding to the cell surface is a rate-limiting step in gene delivery. We determined that the ligand cross-linker spacer length, the conjugate charge ratio, and the number of ligands present affect molecular conjugate binding, internalization, and gene delivery. Furthermore, optimization of these properties significantly enhances conjugate gene delivery efficiency and specificity.

EXPERIMENTAL PROCEDURES

Materials—Mouse EGF, penicillin-streptomycin, L-glutamine, trypsin-EDTA, nonessential amino acids, sodium pyruvate, G418, and minimum Eagle's medium-α were obtained from Life Technologies, Inc. Proteins were iodinated with $Na^{125}I$ (EN Life Science Products) using Iodobeads (Pierce). Unless otherwise mentioned, all other reagents were obtained from Sigma.

Molecular Conjugate Preparation—Streptavidin-polylysine cross-linking and purification were conducted as described previously (18). Three types of biotinylated EGF were synthesized by reacting EGF in HEPES-buffered saline with a 10-fold molar excess of Sulfo-NHS-Biotin, Sulfo-NHS-LC-Biotin, or Sulfo-NHS-LC-LC-Biotin (Pierce) followed by purification on a G-25 column and quantitation by absorbance at 280 nm.

pEGFP-C1 plasmid DNA (CLONTECH) was propagated in the DH5α

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; SA-pK, streptavidin-polylysine.

Escherichia coli strain and purified with a Plasmid Maxi Kit (Qiagen). To form polyplexes, plasmid was added to a 20 mM HEPES (pH 7.0), 1 M NaCl solution at a concentration of 10 μg/ml. Streptavidin-polylysine was added while vortexing, and the resulting complexes were extensively dialyzed against HEPES-buffered saline using 1000 MWCO membrane (Spectrum). Biotin-EGF was then added to the complexes, and the solution was incubated for 1 h.

Determination of Maximum Biotin-EGF Capacity—The maximum valency, the number of biotin-EGF molecules present per plasmid, was determined by incubating streptavidin-polylysine-DNA complexes with radioactively labeled biotinylated EGF. Biotin-EGF, generated with Sulfo-NHS-LC-LC-Biotin, was iodinated to a specific activity of 123,000 cpm/ng and separated from free iodine with a G-25 column. Increasing amounts of the complex were added to 0.165 nmol of radiolabeled biotin-EGF in HEPES-buffered saline and incubated for 1 h. The samples were then centrifuged through 30,000 MWCO Ultrafree-MC centrifugal filter units (Millipore) and washed twice with 0.1% Triton X-100 in HEPES-buffered saline. Radioactivity retained on the filters was counted with a Packard Instruments 5000 series γ-counter.

The efficiency of biotinylation by Sulfo-NHS-LC-LC-Biotin was tested. ^{125}I-Biotin-EGF in phoshate-buffered saline was incubated with streptavidin-agarose for 1 h, both with and without 2 mM free biotin. The agarose was pelleted by centrifugation and washed twice with 4 volumes of 0.1% Triton X-100 in phosphate-buffered saline, and the amount of radioactivity bound to duplicate samples of the agarose was counted.

NR6 Cell Culture—Swiss 3T3-derived NR6 fibroblasts, which lack endogenous EGF receptors, had been transfected with a gene encoding the wild-type human EGFR and selected with G418, as described previously (19). Cells were maintained in minimum Eagle's medium-α supplemented with 7.5% fetal bovine serum, penicillin (100 IU/ml), streptomycin (2.5 μg/ml), 350 μg/ml G418, 2 mM L-glutamine, 100 mM sodium pyruvate, and 10 mM minimum Eagle's medium nonessential amino acids in a humidified incubator (5% CO_2) at 37 °C.

Molecular Conjugate Gene Delivery—For gene delivery experiments, 50,000 cells, counted with a Coulter Electronics Multisizer II, were seeded on 35-mm tissue culture dishes (Corning) and grown overnight. Polyplexes containing 1 μg of DNA were then added to the cells in gene transfer medium, growth medium lacking fetal bovine serum but containing 1% insulin-transferrin-selenium supplement (Life Technologies Inc.) plus 100 μM chloroquine, to yield a total volume of 1 ml. Free EGF (1 μg/ml) was added to some samples. After 6 h, the conjugate solution was aspirated and replaced with regular growth medium. The duplicate samples were trypsinized 48 h later and analyzed for GFP expression using a FACScan flow cytometer (Becton Dickson) with a 488-nm excitation laser and a 530/30-nm fluorescein emission filter.

Cell Surface Binding and Internalization Assays—Radiolabeled conjugates were generated using ^{125}I- streptavidin-polylysine (SA-pK). SA-pK was iodinated to a specific activity of 64,700 cpm/ng of streptavidin and purified with SP Sepharose HP (Amersham Pharmacia Biotech), and 100,000 cpm were added for each microgram of DNA while vortexing. Conjugates were then generated as described above, using biotin-EGF generated with Sulfo-NHS-LC-LC-Biotin. A negligible amount of radioactivity was lost during dialysis.

For binding measurements, subconfluent cells were removed from tissue culture plastic by incubation in 2 mM EDTA for 10 min at 37 °C, centrifuged, and resuspended in gene transfer medium buffered by 20 mM HEPES (pH 7.4). Radiolabeled conjugates containing 0.1 μg of DNA were added to 50,000 cells in a 0.1-ml total volume, and duplicate samples were agitated at 4 °C for 3 h. Cells were then separated from free conjugate using a MultiScreen filtration assay system (Millipore) with 96-well 0.2-μm Durapore filtration plates preblocked with 10 mg/ml bovine serum albumin. After loading of the cell suspensions and application of vacuum, the filters were washed five times with 0.2 ml of ice-cold WHIPS (20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, and 1 mg/ml polyvinylpyrrolidone), and the radioactivity retained on the filters was counted.

For internalization measurements, 100,000 cells were plated on 35-mm dishes and grown overnight. Radiolabeled conjugates containing 1 μg of DNA in 0.1 ml were added with 0.9 ml of gene transfer medium to cells, and duplicate cell samples were incubated for various times in a humidified incubator (5% CO_2) at 37 °C. The conjugate solutions were then aspirated, and the cells were washed twice for 2 min with 1 ml of acidic salt strip (50 mM glycine-HCl, pH 3.0, 2 M NaCl, 1 mg/ml polyvinylpyrrolidone) to remove conjugates bound to the cell surface and the tissue culture plastic. The cells were solubilized with 1 N NaOH, and the internalized radioactivity was counted. Some binding and internalization experiments were conducted in the presence of 1 μg/ml unlabeled EGF, and all data were statistically analyzed by a *t* test.

To determine the effects of multiple biotin-EGF occupancy of a single streptavidin protein on ligand affinity, binding of conjugates to cells was conducted in the presence of biotinylated aprotonin, generated as described above for biotin-EGF, but with a threefold molar ratio of Sulfo-NHS-LC-LC-Biotin. Radiolabeled conjugates at a valency of 15 were generated, and cell surface binding was conducted as described above, but in the absence and presence of excess biotin-aprotonin at a molar ratio of 600 for each plasmid.

Examination of Different Biotinylated EGF Molecules Binding to EGFR—The biotinylated EGF yielding the highest binding of molecular conjugates to EGFR was determined by radioactive competition binding. EGF was iodinated to a specific activity of 160,000 cpm/ng and separated from free iodine with a G-25 column. Cells were seeded at 100,000/35-mm tissue dish and grown overnight. Conjugates were generated with each biotin-EGF at a valency of 10. Duplicate cell samples were incubated in 1 ml of D/H/B (Dulbecco's modified Eagle's medium, 20 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin) with 1 nM ^{125}I-EGF plus conjugates containing 1 μg of DNA. After incubation for 2 h at 4 °C, the cells were washed twice with ice-cold WHIPS. The cells were then solubilized with 1 N NaOH, and the cell-associated radioactivity was counted. Biotin-EGF with the longest spacer arm was used for all other experiments.

Measuring Cell Surface Receptor Levels—Cells were plated at 100,000/35-mm plate and grown overnight. Conjugates were then generated at valencies of 5, 15, and 50. Internalization experiments were conducted with these nonradiolabeled conjugates, as well as with 1 μg/ml EGF, as described above for radiolabeled complexes. At 0, 1, 2, 4, and 6 h, the medium was aspirated, and the remaining surface receptor number was quantified. Briefly, ligand was stripped from the surface with two 2-min washes with 1 ml of ice-cold acidic salt strip, and cells were then incubated in 50 nM ^{125}I-EGF in D/H/B for 3 h at 4 °C. Finally, the duplicate samples were washed three times with 2 ml of ice-cold WHIPS, solubilized with 1 N NaOH, and the radioactivity was counted.

RESULTS

Determination of Maxium Biotin-EGF Capacity—The efficiency of EGF biotinylation by one reagent, Sulfo-NHS-LC-LC-Biotin, was tested by quantifying the binding of radiolabeled biotin-EGF to streptavidin-agarose. 96.2 ± 6.1% of the ligand bound to the gel, while the presence of 2 mM biotin reduced the binding to 4.8 ± 1.2%, where the error is the S.D. of two samples. Under these conditions, the reaction is therefore extremely efficient.

The maximum binding capacity of the conjugates for biotin-EGF was then measured. We synthesized SA-pK at a ratio of 1.5 streptavidin molecules per polylysine chain. Conjugates were then generated at an electroneutral charge ratio and added in increasing amounts to 0.165 nmol of radiolabeled biotin-EGF. Biotin-EGF bound to conjugate samples containing 0, 10, 50, 100, and 200 ng of DNA was separated from free ligand, and the moles of bound biotin-EGF were plotted *versus* the moles of DNA added (data not shown). The data fit the line

$$\text{pmol bound EGF} = 0.13\ \text{pmol} + 63 \times \text{pmol plasmid}\ (R = 0.99) \quad \text{(Eq. 1)}$$

The slope of the line indicates that 63 is the maximum valency, or average number of biotin-EGF molecules that can bind to each plasmid before the streptavidin becomes saturated.

Delivery, Internalization, and Binding as a Function of Conjugate Charge Ratio—Conjugates were generated with various amounts of SA-pK complexed to a fixed amount of DNA, and the effect of the overall charge ratio of these preparations on gene delivery, binding, and internalization was determined. We conducted all experiments in the presence or absence of 1 μg/ml free EGF to compare receptor-specific *versus* nonspecific charge interactions between the conjugates and cells. Fig. 1 shows that gene delivery is negligible at low lysine/nucleotide ratios. All error bars represent the standard deviations of duplicate samples. As the ratio reaches electroneutrality, however, gene transfer efficiency rises steeply in the sample without the excess EGF, reaching 28% at a charge ratio of 4. In the sample with excess ligand, gene transfer rises only for lysine/

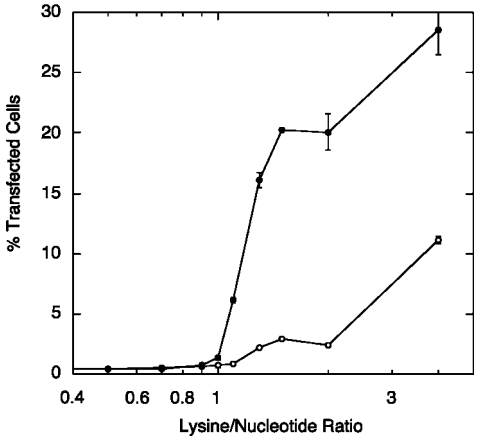

FIG. 1. **Gene transfer efficiency as a function of conjugate lysine/nucleotide ratio.** Gene transfer was conducted using conjugates generated from a various amount of streptavidin-lysine added to 1 μg of DNA/sample, followed by the addition of biotin-EGF at a valency of 10. The percentage of cells expressing GFP after 48 h is plotted *versus* the charge ratio of the conjugate preparation. Samples in the absence (●) and presence (○) of 1 μg/ml free mouse EGF, to block conjugate interactions with the EGFR, were compared.

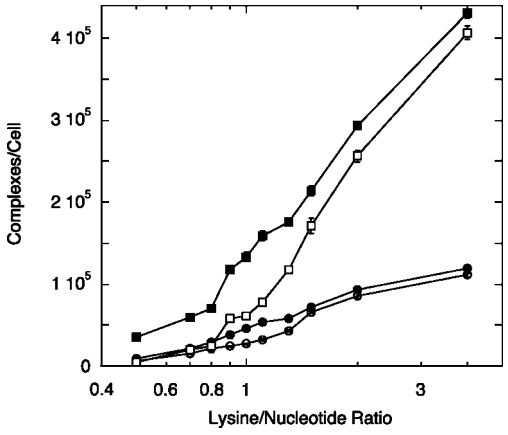

FIG. 2. **Binding and internalization as a function of conjugate lysine/nucleotide ratio.** Specific and nonspecific conjugate binding and internalization were investigated using radiolabeled conjugates generated with various lysine/nucleotide ratios. Binding was conducted for 3 h in cell suspensions at 4 °C and internalization for 6 h with adherent cells at 37 °C, both at a concentration of 1 μg/ml DNA and a valency of 10. The number of plasmids per cell is plotted *versus* the charge ratio. Binding (*circles*) and internalization (*squares*) was compared in the absence (*filled symbols*) and presence (*open symbols*) of 1 μg/ml free EGF.

nucleotide ratios above 1.3, and gene transfer efficiency is statistically significantly lower ($p < 0.05$) in the samples without excess ligand for all charge ratios greater than 0.9. The same trends were observed in CV-1 cells (data not shown).

To determine which step or steps of the gene delivery process can account for the dependence of efficiency on charge ratio, we quantified both conjugate cell surface binding and internalization as a function of lysine/nucleotide ratio. Radiolabeled conjugates were generated by adding a small amount of ^{125}I-labeled SA-pK to conjugate preparations. The association between radiolabeled SA-pK and DNA was stable to the addition of a large excess of polylysine and under the conditions of the incubations with cells (data not shown). Cell binding was conducted in suspension to avoid the large nonspecific, background binding of conjugates to tissue culture plastic. After 3 h of agitation at 4 °C, cells were separated from free conjugate. Assuming the ^{125}I-SA-pK was nearly equally distributed among all plasmids, the approximate number of bound plasmids per cell is plotted *versus* the lysine/nucleotide ratio in Fig. 2. Similar to the trend observed in gene delivery, binding both in the absence and presence of excess ligand rises with increasing charge ratio, from under 10,000 plasmids bound per cell at a lysine/nucleotide ratio of 0.5 to over 110,000 bound at a ratio of 4. The nonspecific binding is statistically significantly lower ($p < 0.05$) than the total binding only between lysine/nucleotide ratios of 0.9 and 1.3.

The same preparations of radiolabeled conjugates were also incubated with adherent cells for 6 h to measure endocytosis. A pH 3, 2 M NaCl wash removed >90% of the radioactivity bound to the cell surface and tissue culture plastic without compromising cell membranes (data not shown). Since chloroquine inhibits lysosomal degradation as well as EGFR recycling (20, 21), the internal counts represent the sum of all endocytosis over the 6 h experiment. The internalization curves, shown in Fig. 2, manifest the same trend as the binding curves, with internalization rising with increasing lysine/nucleotide ratios to a maximum of over 400,000/cell. Although the curves with and without excess ligand are statistically different ($p < 0.05$) at all points, the maximum differences occur near a ratio of 1, as with the binding experiment.

Determination of the Optimal Biotinylated EGF—To determine whether linking EGF to the molecular conjugate reduced its affinity for its receptor, presumably by sterically hindering their interaction, we generated and purified three different biotin-EGF molecules using biotinylation reagents with progressively longer spacer arms. Their ability to mediate specific conjugate binding to the EGFR and gene delivery was then evaluated. The three biotinylated EGF molecules were added at a valency of 10 to electroneutral conjugates, and their affinity for the EGF receptor was gauged by their ability to compete with radiolabeled EGF for binding to NR6 cells. The competition binding results, presented in Table I, are expressed as the percentage by which the binding of ^{125}I-EGF is reduced due to the presence of the conjugates, such that higher percentages indicate better conjugate binding. There is a correlation between spacer arm length and ability to compete in binding, indicating that longer spacer arms between biotin and EGF yield conjugates with a higher affinity for the EGF receptor. Conjugates containing EGF with the 30.5-Å arm reduced binding of 1 nM radiolabeled EGF by 91%, while ones with the 13.5-Å arm inhibited binding by only 10%. Gene transfer was then conducted, and at a valency of 10, increased binding improved gene delivery efficiency (Table I). The conjugates generated with the smallest linker had an efficiency of 2.3%, while the longest linker yielded an 18% efficiency. Therefore, extending the spacer arm between EGF and biotin improves cell surface binding and gene delivery efficiency at low valency.

Delivery, Internalization, and Binding as a Function of Conjugate Valency—Conjugates at a lysine/nucleotide ratio of 1 were generated, and varying amounts of biotin-EGF with the longest spacer arm were added. Fig. 3 shows that the gene transfer efficiency rises sharply at low valencies, peaks at 18% between a valency of 10 and 20, and then declines to below 2.5% between 20 and 50. The same trend was observed in CV-1 cells (data not shown). To investigate what could account for this behavior, binding and internalization experiments were conducted with varying valencies, and the results are shown in Fig. 4. Binding and internalization are both maximal at the same valencies as gene delivery, between 10 and 20, with binding peaking at 72,000 plasmids per cell and internalization at 137,000.

Reduced binding, internalization, and delivery at higher valencies could potentially be due to steric hindrance between multiple biotin-EGF proteins bound to the same streptavidin. To address this possibility, we measured binding of conjugates

TABLE I
Effects of EGF cross-linker spacer arm length

To measure effects on specific conjugate binding to the EGFR, three biotin-EGF molecules were generated with increasing spacer arm lengths between the biotin and EGF, shown in the second column. Conjugates, generated with each biotin-EGF at a valency of 10, were assayed for binding to the EGFR and gene delivery. In a competition experiment, the percentage by which the binding of free ^{125}I-EGF to cells was reduced due to the presence of the conjugate is shown in the third column. The percentage of cells expressing GFP after gene transfer by each is shown in the fourth column.

Crosslinker name	Spacer arm length	Inhibition of EGF binding	Gene transfer efficiency
	Å	%	%
Sulfo-NHS-Biotin	13.5	10.4 ± 5.8	2.3 ± 0.4
Sulfo-NHS-LC-Biotin	22.4	17.8 ± 5.5	6.9 ± 5.5
Sulfo-NHS-LC-LC-Biotin	30.5	91.0 ± 0.5	18.0 ± 1.7

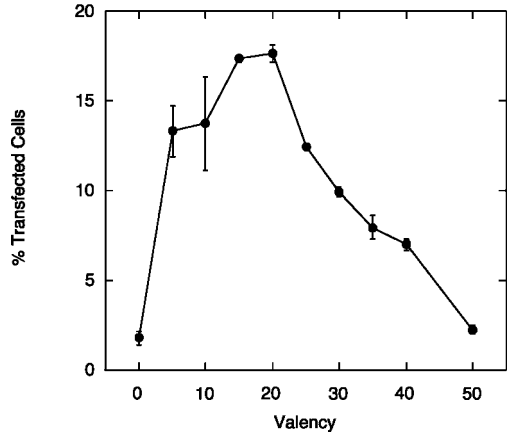

FIG. 3. **Gene transfer efficiency as a function of conjugate valency.** Gene transfer to NR6 cells was conducted using conjugates generated with a varying amount of biotin-EGF added to conjugates at a lysine/nucleotide ratio of 1, and 1 μg of DNA was added per sample. After 48 h, cells were analyzed for GFP expression by flow cytometry, and the percentage of green fluorescent cells is plotted *versus* valency.

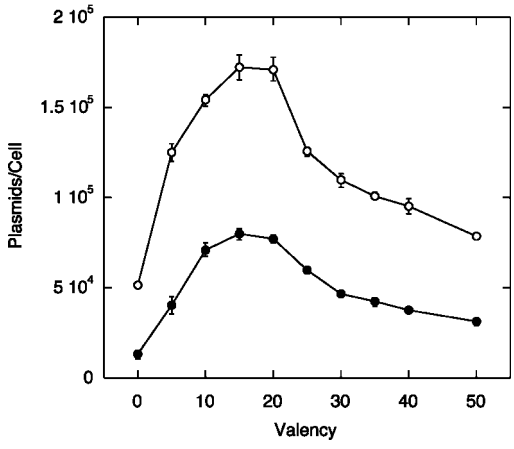

FIG. 4. **Conjugate binding and internalization as a function of conjugate valency.** Conjugate binding and internalization by NR6 cells was investigated using conjugates generated with various valencies, or biotin-EGF/plasmid ratios. Experiments were conducted as in Fig. 2, and the number of plasmids bound (●) and internalized (○) per cell is plotted as a function of valency.

at an EGF valency of 15 in the presence of a large excess of biotinylated aprotonin, which is of similar size to EGF, to saturate unoccupied streptavidin sites. The presence of biotin-aprotonin made no statistically significant difference in conjugate binding to cells (data not shown). Therefore, steric interference between multiple biotin-EGF molecules likely does not account for the observed drops in delivery, binding, and internalization at high valency.

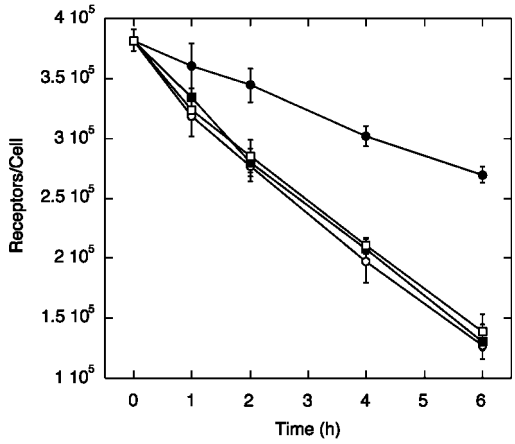

FIG. 5. **Dependence of receptor down-regulation on conjugate valency.** EGF receptor down-regulation due to internalization of molecular conjugates was examined. Conjugates were generated at valencies of 5, 15, and 50. These samples, as well as a saturating concentration of free EGF, were incubated with cells for varying periods of time, and surface receptor number was then quantified by binding of a saturating amount of radiolabeled EGF. The number of remaining surface receptors is plotted *versus* conjugate valency for conjugates of valency 5 (●), 15 (○), and 50 (■), as well as for 1 μg/ml EGF (□).

Another possibility that could explain the reduction in conjugate binding observed at higher valencies is that the receptors become saturated. Multiplying valency by the number of plasmids bound per cell shows that, at valencies of 15 or greater, there are roughly one million EGF molecules attached to conjugates bound to each cell. Even if only a fraction of the conjugate surface area is close enough to the cell membrane for the ligands to bind, at valencies of 15 and above they likely occupy nearly all the approximately 380,000 receptors per cell (see below). Therefore, as valency increases and each conjugate binds a larger number of the receptors, the number of bound conjugates decreases.

Measurement of Cell Surface EGF Receptor Levels—The reduction in the number of conjugates bound at high valencies may lead to reduced conjugate internalization and gene delivery. If conjugates of higher valencies occupy nearly all the surface receptors, then the specific receptor-mediated endocytosis is likely saturated, as has been observed in NR6 and other cells at higher levels of EGF receptor occupancy (22, 23). With endocytosis saturated, the cells would be specifically internalizing the highest number of receptors possible, but at high valencies, each conjugate binds to a large number of these receptors. Therefore, as valency is increased the fixed, saturated flux of receptors into the cell would be able to carry along progressively fewer conjugates.

To test the possibility that conjugates saturate receptor internalization at higher valencies, cells were incubated with conjugates of valency 5, 15, or 50, or with 165 nM EGF, for various times up to 6 h. The ligand was then stripped from the cell surface, and the remaining number of surface EGFR was measured. A saturating level of free EGF should lead to the maximum potential rate of receptor internalization. In addition, the presence of chloroquine inhibits EGFR recycling after endocytosis (20, 21); therefore, the rate of disappearance of cell surface EGF receptor due to the presence of 1 μg/ml EGF plus 100 μM chloroquine should theoretically be the maximum possible surface receptor down-regulation rate. Fig. 5 shows that, with excess free EGF, the surface receptor number decreases approximately linearly from 380,000/cell to 180,000/cell by 6 h, or only 43 ± 5.5% of its original value, where the error is the S.D.s of two samples. At a conjugate valency of 5, receptors are

down-regulated to only 71 ± 3.4%, or 270,000 receptors/cell. However, at valencies of 15 and 50, EGF receptors are down-regulated to 40 ± 1.8% and 42 ± 8.4% of their original levels. The receptor down-regulation due to conjugates at valencies of 15 and 50 is not statistically distinguishable from that observed for free EGF.

DISCUSSION

We have investigated whether polyplex interactions with the cell surface can be a rate-limiting step for gene delivery, and if so, whether this barrier can be overcome to improve gene delivery efficiency. We focused on three conjugate parameters that could potentially affect binding to the cell, the lysine/nucleotide ratio, the cross-linker used to attach the ligand, and the number of ligands added per plasmid. The effects of these properties on cell surface binding, internalization, and gene delivery have been examined. For these studies, we employ an experimental system that uses EGF to direct delivery of plasmid DNA encoding GFP to mouse fibroblasts stably expressing the human EGFR.

Zeta potential measurements show that adding increasing amounts of polycation to a fixed amount of DNA changes the surface charge of polyplexes from negative, through electroneutral, to positive (24, 25). The electropositive conjugates nonspecifically bind to the negatively charged cell surface, likely due to interactions with proteoglycans (17). Therefore, if specific, receptor-mediated gene delivery is desired, it is important to know precisely how this nonspecific binding depends on conjugate charge. The effects of the lysine/nucleotide ratio have been examined in other systems, and increasing the proportion of polycation increases overall gene delivery (4, 26, 27). However, the interpretation in most cases is complicated by the fact that as more cross-linked ligand-polylysine was added to the DNA, the number of ligands increased along with the charge ratio. In addition, the contributions of receptor-ligand *versus* nonspecific ionic interactions to gene delivery were not resolved.

We measured the relative contributions of specific and nonspecific interactions between conjugates and cells to binding, internalization, and gene delivery as a function of the lysine/nucleotide ratio. Binding and internalization increased as the lysine/nucleotide ratio was increased from 0.5 to 4, as shown in Fig. 2. The internalization curves are steeper than the binding curves because, with inhibition of recycling and degradation by chloroquine, they represent an accumulation of binding and internalization events over the 6 h. The curves in the absence and presence of excess free ligand are similar at all charge ratios except near a lysine/nucleotide ratio of 1. Below this ratio, regardless of the presence of biotin-EGF, the negatively charged conjugates are probably ionically repelled by the cell membrane or not sufficiently condensed to be internalized. Conjugates generated at a ratio greater than 1 likely nonspecifically adsorb to the surface, again independent of biotin-EGF. Only between ratios of 0.9 and 1.3, where charge effects are likely at a minimum, are receptor-mediated binding and internalization larger than the nonspecific contributions. Our results are consistent with the zeta potential measurements of Wolfert *et al*. (24), who show that their polylysine molecular conjugates have a neutral surface charge at a lysine/nucleotide ratio of approximately 1.2. Even electroneutral complexes, however, have surprisingly significant nonspecific binding and internalization.

Gene transfer efficiency as a function of charge ratio, shown in Fig. 1, follows similar trends to binding and internalization; delivery is always negligible at low ratios, and nonspecific delivery increases at high ratios. However, in contrast to binding and internalization, at ratios above 1 gene delivery is always much higher when receptor-ligand interactions are not blocked, a result that implies that receptor-ligand binding may play an important role even after entry into the cell. One possible explanation for the observation is that conjugates bound to EGF receptors are sorted differently within endosomes, since it is known that ligands undergo different endosomal trafficking fates depending on whether they remain associated with the EGFR (28). Alternatively, there are numerous reports of EGFR translocation to the cell nucleus in a number of cell types including NR6 cells, so it is conceivable that the receptor could facilitate nuclear delivery of conjugates (29–32). In any case, however, if highly receptor-specific gene delivery is desired, even at the expense of efficiency, then conjugates must be generated close to electroneutrality.

We have also investigated the effects of the overall affinity of polyplexes for a cell surface receptor on gene delivery. This affinity can be altered in two ways, by changing the properties of individual ligands, or by varying the number of ligands present per conjugate. We found that lengthening the cross-linker arm used to tether EGF to the conjugate significantly enhances the specific binding of conjugates to the EGFR. The nonspecific binding, determined by the conjugate charge, should not be affected by the cross-linker. Biotin dimers cross-link streptavidin only when the two biotins are separated by at least 23 Å (33), so that we would expect that the minimum spacer length for effective attachment of biotin-EGF to the conjugates is 11.5 Å. All spacers we used are longer, so the additional space may provide the flexibility required for EGF to bind to its receptor in the correct orientation. This improved binding with longer spacers translates into more efficient gene delivery at low valency. The result that increasing spacer arm length significantly improves binding and gene expression has also been observed with retargeted retroviral vectors displaying ligands fused to an envelope glycoprotein (34, 35).

While the results with different cross-linkers showed that increasing affinity at low valency improves gene delivery, the biphasic dependence of gene delivery on valency demonstrated that increasing affinity too high actually significantly hampers gene delivery. At low valency, inefficient conjugate binding results in low internalization and gene delivery. Increasing valency improves binding, internalization, and delivery to an optimum at a valency near 15. Further valency increases, however, lead to a rapid decrease in all three curves and an 8-fold drop in efficiency. Binding likely decreases because, from valencies of 15–50, nearly all the 380,000 EGF receptors may be occupied. This conclusion is supported by our earlier analysis that for valencies of 15 or greater the number of biotin-EGF molecules attached to the conjugates bound to each cell roughly plateaus near 1 million. In addition, conjugates of valency 15 reduce the binding of 1 nM radiolabeled EGF by as much as 91% (Table I). With the receptors saturated, increasing the number of ligands per plasmid only decreases the number of conjugates that can bind each cell.

If a high number of EGF receptors is occupied, then EGF receptor-mediated endocytosis becomes saturated, meaning that the rate of receptor internalization reaches a maximum. Endocytic saturation, which occurs at an EGFR occupancy of 50,000 to 100,000 for NR6 cells,[2] is probable with higher valency conjugates. To test this possibility, we monitored the disappearance of EGFR from the cell surface. Conjugates of valency 5 down-regulate the EGFR by only 29% over 6 h. In contrast, conjugates of valency 15 and 50 down-regulate the receptor by over 65%, the same result observed with 165 nM free EGF. Therefore, conjugates of valency 15 already drive the highest rate of receptor endocytosis, so increasing valency fur-

[2] J. Haugh, personal communication.

ther only decreases the number of plasmids carried inside the cell by the fixed number of receptors internalized. In summary, at low valency, receptors are inefficiently utilized because not enough are occupied and internalized, and at high valency, receptors are inefficiently utilized because each conjugate binds too many. This finding should be relevant not only to molecular conjugates, but also to other targeted gene delivery systems such as immunoliposomes or engineered viral vectors where the valency can be varied, particularly when a high affinity ligand such as a growth factor or antibody is employed (36–41).

These experiments show that binding to the cell surface is a barrier to gene transfer, and that the conjugate can be engineered to increase efficiency and specificity. We find there is a relatively narrow window of charge ratios in which the receptor-specific is significantly greater than the nonspecific binding, internalization, and delivery. In addition, there is an optimal conjugate-receptor affinity for gene delivery. At low valency, affinity is insufficient for effective binding and internalization. However, increasing valency beyond the optimum significantly decreases gene delivery due to saturation of binding and internalization. These fundamental results of this model system can potentially be applied to other targeted synthetic or viral gene delivery systems. In summary, a quantitative examination of the mechanism of receptor-mediated gene delivery can yield improvements in gene transfer efficiency and specificity.

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