Epidermal Growth Factor Receptor-Mediated Gene Delivery:
A Model System for Engineering Selective Gene Therapy Approaches

by

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ABSTRACT

The most significant challenge facing efforts at gene therapy is the development of efficient gene delivery vehicles. Most efforts are directed at improving viral vectors, which are currently limited by relatively low efficiency and problems with toxicity and immunogenicity. A second approach is to develop synthetic vectors that adopt many strategies viruses have evolved to deliver genes, but exclude harmful viral materials. One such vector, the polyplex or molecular conjugate, has significant potential for targeted, *in vivo* delivery; however, its efficiency must first be substantially improved. Polyplexes are generated by condensing nucleic acids with a polycation that can be linked to a ligand to mediate targeted gene delivery. We have developed a novel polyplex system that employs epidermal growth factor to direct the delivery of DNA encoding the green fluorescent protein to various cell lines. Furthermore, we have used this system to examine the mechanism of conjugate gene transfer in order to identify rate-limiting steps and obtain information on how to enhance delivery efficiency. We have found that two steps, cell surface binding and intracellular conjugate unpackaging, are barriers to delivery. Nonspecific cell surface binding can compromise delivery specificity, and saturation of receptor binding and internalization can limit efficiency. In addition, a failure of the polycation to dissociate from DNA in the nucleus can limit gene expression. By engineering conjugates to overcome these barriers, however, we enhance delivery specificity and improve efficiency by nearly two orders of magnitude.

Thesis Supervisor: Douglas A. Lauffenburger
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1. Background

1.1 Origins of Gene Therapy

Cultures around the world have used medicine to treat illness for thousands of years. During the 19th century, the science of pharmacology was born when it became recognized that the purification of drugs facilitates their scientific study and clinical application. This approach that led to the development of early anesthetics and antibiotics (Weatherall, 1993), small organic compounds that exert their effects by targeting and altering the activity of proteins. In the 1920s, insulin was purified and soon used to treat diabetes, marking the first clinical use of a protein itself as the therapeutic agent (Abel *et al.*, 1927; Banting and Best, 1922). Finally, the last two decades have witnessed extensive investigation of the vast therapeutic potential of the substance that encodes the proteins: genes.

Gene therapy, or the introduction of genetic material to the cells of individuals for therapeutic benefit, has conceptual origins in fundamental investigations of gene transfer. In 1944, using Pneumococcal bacteria, Avery, MacLeod, and McCarty became the first to demonstrate that "inheritable and specific" changes in cell behavior can be induced by the transfer of a gene contained within DNA from one organism to another (Avery *et al.*, 1944). Viruses were soon shown to have the ability to transmit genes to both bacteria and animals (Sambrook *et al.*, 1968; Zinder and Lederberg, 1952). Subsequent studies demonstrated that complexation of nucleic acids with a variety of compounds, such as calcium phosphate, spermine, or DEAE-dextran, yielded "synthetic" vehicles that transferred genes to cells in culture (Dubes and Klingler, 1961; Graham and Van Der Eb, 1973; Smull and Ludwig, 1962; Vaheri and Pagano, 1965).

These early successes rapidly led to the idea that gene transfer could be applied to medicine:

"We can ... be somewhat optimistic on the long-range possibility of therapy
by the isolation or design, synthesis, and introduction of new genes into
defective cells of particular organs." (Tatum, 1966)

It was quickly recognized that the natural ability of viruses to deliver nucleic acids could be
exploited by constructing an artificial virus, or viral vector, to deliver a therapeutic gene.
However, progress in gene therapy would have to wait for techniques that permitted the
isolation and incorporation of specific genes into delivery constructs:

"Once the essential techniques for grafting segments of DNA from different
sources onto that of a microbe have been perfected, experiments along these
lines provide the most favourable opportunity to select those segments of
DNA information which are needed." (Wolff and Lederberg, 1994)

Recombinant DNA technology, or the ability to cut and graft or recombine specific DNA
sequences, was developed in the early 1970s (Cohen *et al.*, 1973; Jackson *et al.*, 1972).
This ability to recombine DNA was soon applied to develop viral vectors, when Berg *et al.*
developed a series of recombinant simian virus (SV40) vectors that carried bacterial or
phage DNA and maintained the ability to replicate in mammalian cells in culture. Soon
afterwards, in 1979, Mulligan *et al.* infected cultured monkey kidney cells with an SV40
vector carrying the rabbit β-globin gene and thus became the first to express a foreign
protein in a mammalian cell (Goff and Berg, 1976; Jackson *et al.*, 1972; Mulligan *et al.*,
1979).

Before viral vectors could be applied to gene therapy, a method to produce high
titers of safe, replication-incompetent vectors was needed, and in 1983 the retroviral
packaging cell line system was developed to meet these requirements. This method has
two components: a packaging cell line and a viral vector (Mann *et al.*, 1983; Miller, 1990).
The cell line is stably transduced with genes encoding all the viral structural and enzymatic
proteins necessary for viral replication and assembly, but cannot produce infectious
particles. The vector contains the transgene and a genetic signal required for packaging into
a virion, but cannot be replicated unless it is introduced into the packaging line. By

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separating the viral genome into two pieces, the packaging cell line system can produce high titers of recombinant vector while minimizing the possibility of generating wild-type infectious virions.

1.2 Potential Disease Targets for Gene Therapy

As progress in gene delivery brought investigators closer to a gene therapy attempt, they were also considering strategies for treating a variety of diseases. The advent of molecular genetics has led to the characterization of diseases on a molecular level, facilitating the identification of genes whose introduction into cells could potentially cure specific disorders. Three major classes of diseases are candidates for treatment by genetic therapy: inherited single gene disorders, cancer, and AIDS.

Mendelian Inheritance in Man lists over 4000 classically-inherited genetic disorders (McKusick, 1991). The basis of such a disorder was first identified as a gene mutation when it was discovered that familial hypercholesterolemia is caused by a mutation in the low-density lipoprotein receptor (Brown and Goldstein, 1976). A number of other single gene disorders has subsequently been characterized, including adenosine deaminase (ADA) deficiency and cystic fibrosis, leading to the possibility that these diseases can be cured by the introduction of a correct version of the defective gene into the appropriate cells (Morgan and Anderson, 1993).

A number of strategies are also being developed to treat cancer. These include increasing the immunogenicity of a tumor to stimulate an immune response, enhancing cells of the immune system to improve their response, introducing a suicide gene to kill tumor cells, inhibiting the expression of an oncogene, or increasing the expression of a tumor suppresser (Bertelsen *et al.*, 1995; Culver and Blaese, 1994). Among the potential approaches for treating AIDS are the expression of soluble decoy viral receptors, dominant negative HIV gene mutants, and antisense RNA or ribozymes to reduce the levels of viral RNA (Gilboa and Smith, 1994).

1.3 Early Clinical Efforts in Gene Therapy

In 1990, Blaese and Anderson became the first to initiate a gene therapy clinical trial (Blaese *et al.*, 1995). The two patients suffered from ADA deficiency, a condition where the lack of the enzyme ADA leads to the buildup of a toxic metabolite that kills T lymphocytes and thus causes severe combined immunodeficiency. Although they had not responded well to PEG-ADA, this traditional protein replacement therapy was continued throughout the trial. At various times during the protocol, lymphocytes were isolated from the patents' blood, propagated in culture, and infected with a retroviral vector encoding ADA. After further expansion in culture, the cells were reintroduced into each patient.

The study had several positive outcomes. First, gene transfer did occur since the transgene was detected by PCR in the patients' T cells three years after the initiation of the trial. Second, retroviral gene transfer did not harm the patients. Third, after gene transfer investigators observed an increase in ADA activity that correlated with some improved responses to antigen challenge. The study also highlighted several major problems. Gene transfer was inefficient, particularly in one patient. In addition, the increases in ADA levels, T cell numbers, and immune response were erratic, transient, and inconsistent between patients. Finally, since patients were initially not removed from PEG-ADA treatment, it is unknown whether the ADA generated due to the gene therapy alone would have been sufficient for any response.

1.4 Emerging Problems in Gene Therapy

Although the ADA clinical trial demonstrated the safety of gene therapy, a number of deep and significant problems were revealed by this and the many subsequent clinical trials. Therefore, a consensus began to develop that despite the strong medical and commercial imperatives to initiate more clinical trials, an extensive effort to solve these problems by focusing on the basic science of gene therapy was needed before clinical trials

could succeed (Hodgson, 1995; Hodgson, 1995; Marshall, 1995; Orkin and Motulsky, 1995).

There are two major classes of gene delivery, *ex vivo* and *in vivo*, and the problems encountered depend somewhat on what type is used. In *ex vivo* gene therapy like the ADA trial, cells are removed from the patient or donor, transduced with the therapeutic gene, and reintroduced into the individual. For tissues such as brain or lung that cannot be removed, expanded *in vitro*, and replaced, the vector can also be introduced directly into the patient, a method known as *in vivo* gene therapy. By far the most significant obstacle that faced both methods was that gene delivery was too inefficient to lead to the levels of transgene expression required to cure a disorder, the problem that is the focus of this thesis. In addition, vectors should be safe, meaning that replication-competent virus should not be generated from viral vectors, and no vector should be toxic to target cells. Furthermore, vectors should be able to incorporate large genetic insert sizes, to allow for the incorporation of large regulatory regions, coding regions, or multiple genes.

Another problem facing both *ex vivo* and *in vivo* gene delivery is the duration of expression in target cells after introduction of the transgene. Although many applications require sustained expression, it is often transient and unstable. Also, certain vectors or the proteins they produce can elicit an immune reaction which can eliminate transgene expression. Not only does such a reaction contribute to the problem of transgene expression, but it can also pose a danger to the patient.

One challenge that is unique to *in vivo* gene delivery is the development of vectors which can target gene delivery and/or expression to a specific population of cells (Glaser, 1994). In addition, while *ex vivo* gene therapy is typically implemented on dividing cells in culture, in some cases *in vivo* gene therapy must target nondividing cells, which present more of a challenge. Significant efforts are directed at overcoming each of these problems to reach the ultimate goal in gene therapy: the development of an efficient, targeted, injectable vector.

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1.5 Gene Delivery Vectors

The two major types of gene delivery vehicles are viral and synthetic vectors, and each vector has characteristic advantages and disadvantages that make it suitable for particular applications. As shown if Figure 1.1, all vectors facilitates the passage of nucleic acids from the cell surface to the nucleus through a series of similar steps. First, a vector binds to the cell surface, either through nonspecific charge interactions with the negatively charged cell surface membrane or by specifically binding to a cell surface receptor. Vectors that bind to a receptor have the potential for targeted gene delivery to only those cells that express the receptor. In the second step, some vectors enter the cell by direct disruption or fusion with the cell surface. Alternatively, the vector is internalized and routed to endosomal vesicles, and once in the endosome the nucleic acid escapes to the cell cytoplasm. The interior of endosomes has a pH of approximately 5 or 6 (Mellman *et al.*, 1986; Sorkin *et al.*, 1988), and the mechanism for vector escape from the endosomes is often triggered by this acidity. Finally, for most vectors the nucleic acid is eventually transported to the nucleus, where the delivered genes are expressed.

1.5.1 Viral Vectors

As shown in Figure 1.2, viral vectors are generated by excising genes from a wild-type virus to render it replication-defective and to free space for the insertion of the transgene. The genes required for viral replication are then supplied in trans by packaging cell lines or helper viruses in order to produce recombinant vector. In addition, second generations of viral vectors can be generated by excising additional viral genes in order to reduce problems with toxicity or immunogenicity and to free more space for the transgene. The major viral vectors under development are based on retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses.

1.5.1.1 Retroviral Vectors

Most retroviral vectors are based on the Moloney murine leukemia virus (Mo-MLV), but all share a similar infection pathway. After the virion encounters a target cell surface, the viral attachment protein (VAP) mediates the binding of the virion to a specific cell surface receptor. The Mo-MLV receptor is a cationic amino acid transporter expressed on most cell types, but other retroviral receptors may be expressed on more restricted cell populations (Cosset et al., 1995; Wang et al., 1991). After the virion attaches to the surface, its envelope fuses with the cell membrane, allowing the viral capsid to enter the cell cytoplasm. Viral reverse transcriptase then reverse transcribes the viral RNA into DNA, which is transported into the nucleus and randomly integrated into the chromosomal DNA. Genes within the integrated DNA are then expressed (Boris-Lawrie and Temin, 1994).

The retroviral vector has several significant advantages. First, because it mediates the integration of a transgene into the host cell chromosome, the transgene theoretically has the potential for expression throughout the life of a cell. In addition, Mo-MLV can deliver genes to a wide variety of cells, making it well-suited for *ex vivo* gene delivery. Furthermore, since all viral genes are eliminated from Mo-MLV vectors, the chance of virus-specific immune reaction against transduced cells is substantially reduced. However, one drawback of most retroviral vectors is that the gene transduction efficiency, the number of gene copies delivered per target cell, is relatively low (Le Doux and Yarmush, 1996). Also, the random proviral integration is potentially dangerous, since the integration could interfere with a vital cell gene or even activate an oncogene. In addition, Mo-MLV cannot infect nondividing cells and therefore cannot be used to deliver genes to terminally differentiated cells, such as neurons or cardiac myocytes (Leiden and Barr, 1994; Roe *et al.*, 1993). Furthermore, delivery of more than 9 kb of genetic material is not possible. Finally, since the Mo-MLV receptor is expressed on most cell types, targeted *in vivo* gene delivery is difficult (Cosset *et al.*, 1995).

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Vectors and infection protocols have been improved in various ways to overcome some of these disadvantages. For example, *ex vivo* transduction efficiency can be enhanced by using centrifugation or convection to concentrate virions close to the cell surface and thereby overcome a diffusional limitation (Bahnson *et al.*, 1995; Chuck and Palsson, 1996). Additionally, gene expression levels can be significantly improved by making modifications to regulatory and control regions within the vector backbone (Robbins *et al.*, 1997). Furthermore, there have been a number of attempts to target retroviral gene delivery by fusing a ligand onto a viral envelope glycoprotein. All resulting vectors have the capacity to specifically infect cells expressing a receptor for the ligand, though efficiencies vary widely (Cosset *et al.*, 1995; Han *et al.*, 1995; Kasahara *et al.*, 1994; Valsesia-Wittmann *et al.*, 1996).

A final limitation of Mo-MLV vectors that can be overcome is their inability to infect nondividing cells, since vectors can be generated from a retrovirus known to infect proliferating cells: HIV. Lentiviral vectors have been shown to efficiently transduce terminally differentiated cells such as macrophages and neurons (Naldini *et al.*, 1996; Naldini *et al.*, 1996; Zufferey *et al.*, 1997). Since the safety of using a vector derived from the virus that causes AIDS is an obvious concern, investigators have established that attenuated vectors in which as many as five of the nine genes present in the parental virus are deleted are still able to efficiently deliver genes to neurons *in vivo* (Naldini *et al.*, 1996; Zufferey *et al.*, 1997).

1.5.1.2 Adenoviral and Adeno-associated Viral Vectors

Although retroviral vector chromosomal integration and relatively low efficiency make the traditional Mo-MLV vectors very well-suited for *ex vivo* delivery, *in vivo* gene delivery has the advantages that it is a simpler procedure, appropriate for a wider variety of tissues, and less expensive. Adenoviral vectors have strong potential for efficient *in vivo* gene delivery to a wide variety of dividing or nondividing cells, but can be significantly

limited by problems with toxicity and immunogenicity (Brody and Crystal. 1994; Kozarsky and Wilson, 1993; Wilson, 1996).

The adenovirus is composed of 36 kDa linear, double-stranded DNA surrounded by a capsid but no envelope. After the fiber protein of the capsid mediates binding to the primary adenoviral receptor on cell surfaces, the virion is internalized through interaction with a second cell receptor, an integrin (Bergelson et al., 1997). Once inside, the virion progressively uncoats, culminating in the delivery of the DNA to the cell nucleus (Greber et al., 1993). Within the nucleus, the viral genome can possibly be stably maintained episomally, potentially due in part to the activity of the viral preterminal protein encoded in the E2 region (Lieber et al., 1997).

Adenoviral vectors have the significant advantage that they are the most efficient vectors for gene delivery to a wide variety of dividing or nondividing cell and tissue types in vitro and in vivo. Also, their genome is larger than that of retroviral vectors and therefore has the potential to carry larger inserts after viral genes are deleted. Finally, their potential for maintenance as a stable episome avoids the problems of random chromosomal integration associated with retroviruses.

However, the most significant problem of adenoviral vectors is that they elicit an immune response that leads to toxicity, elimination of transgene expression, difficulty with vector readministration, and even potential danger to a patient. The immune reaction can be directed against the adenoviral vector itself. For example, intratracheal administration of large doses of incomplete or inactivated vector leads to significant pulmonary inflammation in mice (McCoy et al., 1995). In addition, the first generation of viral vectors still contained a large number of viral genes, and their expression by transduced cells elicited an immune response that often led to the elimination of transgene expression. However, further study of adenoviral vector biology led to the identification of viral genes that could be deleted without compromising delivery efficiency. The second generation of vectors developed in these studies elicited a weaker immune response and could therefore be stably

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maintained for weeks in the mouse liver (Gao *et al.*, 1996; Lieber *et al.*, 1997). Vectors have also been engineered for specific gene delivery, since modifications to the virion fiber protein can generate vectors with specificity for different primary receptors (Douglas *et al.*, 1996; Wickham *et al.*, 1996).

Another vector with promise is based on adeno-associated virus (AAV), a 4.7 kb single stranded DNA virus (Carter, 1996; Fisher *et al.*, 1996; Rolling and Samulski, 1995). As its name suggests, AAV is a defective virus that cannot replicate in the absence of a helper virus, such as adenovirus or herpes virus. Depending on the host cell's physiological state, the AAV genome can integrate into a specific region of human chromosome 19. This capacity for targeted chromosomal integration makes AAV attractive for delivery of transgenes that are maintained for the life of a target cell and avoids the danger of random integration associated with retroviral vectors. Additionally, AAV is not a pathogenic virus, a fact that improves the safety of AAV vectors.

AAV vectors have delivered genes to a variety of dividing or nondividing cells in culture with varying efficiencies. *In vivo* studies have used AAV to deliver transgenes to a number of tissues in various animal models, and expression has persisted as long as 18 months in muscle. These studies have shown no evidence of an inflammatory or toxic response, although neutralizing antibodies can develop and prevent expression from further viral inoculations. Based on these results, clinical trials have been initiated to test delivery of CFTR cDNA to cystic fibrosis patients.

Despite its potential, a number of problems with AAV still remain. First, the small genome limits the size of a transgene insert. Second, depending on the physiological state of the host cell, AAV can either integrate or exist episomally, and the regulation of this choice is not yet understood. Furthermore, with vectors deleted for one viral gene, *rep*, integration appears to be less efficient and and specific to chromosome 19. The promise of safe delivery and targeted integration, however, will motivate efforts to overcome these problems.

1.5.1.3 Other Viral Vectors

There are a number of other vectors under development, including ones based on SV40 (Madzak *et al.*, 1992), vaccinia (Moss, 1992), and Sindbis viruses (Ohno *et al.*, 1997), as well as hybrid viral vectors that combine the advantageous features of two viruses (Feng *et al.*, 1997; Fisher *et al.*, 1996; Johnston *et al.*, 1997). One notable vector which can mediate efficient delivery to nondividing cells is the herpes simplex 1 (HSV-1) vector. HSV is a large, enveloped virus with a double stranded, 152 kb DNA genome encoding more than 80 genes. One advantage of HSV-1 vectors is that the parent virus is able to establish a latent infection in which the genome is stably maintained without replication in nondividing cells. HSV-1 vectors are generated by deleting essential viral genes and thereby forcing the virus into latency, and the resulting vectors can express a transgene for weeks in a nondividing cell, making them particularly useful for delivery to neurons. Furthermore, new vectors deleted of additional viral genes can be generated and potentially have fewer of the cytotoxicity problems associated with the first generation of vectors (Fink *et al.*, 1996; Rasty *et al.*, 1997).

1.5.2 Synthetic Vectors

The work reviewed above establishes that although viral vectors have the potential for efficient and sustained expression *in vivo*, progress must still be made to develop newer generations of vectors that can overcome problems of toxicity, immunogenicity, target cell specificity, and stability of expression. An alternative to viral vectors are synthetic delivery vectors, which are based on the principle that by assembling a vector from individual, synthetic components that lack any harmful viral materials, the liabilities of some viral vectors can be avoided.

Figure 1.2 shows the most common pathways in the "evolution" of synthetic vectors. The most synthetic vector is naked plasmid DNA, which is surprisingly efficient in delivering genes to some cell types, particularly muscle (Dowty and Wolff, 1994).

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Along one evolutionary path, lipids are added to the DNA to generate liposomes or lipoplexes, conceptually similar to enveloped viruses. Ligands, similar to viral attachment proteins, can then be added to the liposome to target gene delivery to cells expressing a specific receptor. Along a second path, a cationic polymer, or polycation, is added to condense the DNA and generate a molecular conjugate or polyplex, conceptually similar to . the packaging of viral genomes by capsid proteins. A ligand can likewise be attached to the polyplex for targeted delivery. Thus, synthetic vectors borrow technology from viruses in order to increase gene delivery efficiency, and the lack of viral genes or proteins typically makes them less toxic and immunogenic. One could speculate that as synthetic vectors borrow more strategems from viruses, and viruses are stripped of more of their genes, these two lineages may merge with the development of an efficient synthetic virus that bears only a small resemblance to either of its two origins. However, the current generations of synthetic vectors are still hampered by low gene delivery efficiency. In addition, gene expression after delivery is typically transient.

1.5.2.1 Lipoplexes

Liposome or lipoplex vectors, generated by complexing nucleic acids with lipids, are classified into two major categories by the type of lipid used: anionic and cationic liposomes. During gene delivery, early generations of liposomes nonspecifically adsorbed to the cell surface, and after internalization, the lipids mediated the disruption of the endosomal membrane to release the DNA into the cytoplasm. A fraction of the DNA is then transported to the nucleus. This synthetic gene delivery construct has the potential to overcome some shortfalls of viral delivery, but is limited by low gene transfer efficiency. In addition, gene expression after delivery is often transient.

Anionic and cationic liposomes deliver DNA by distinct mechanisms, and strategies or modifications to improve the delivery efficiency of each are therefore different. Anionic, or pH sensitive liposomes, have the advantage that the low endosomal pH catalyzes a phase

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transition of the vector's lipids that results in the disruption of the endosomal membrane, which otherwise poses a major barrier to gene delivery (Lee and Huang, 1996). A major problem with earlier attempts at delivery by anionic liposomes, however, was that they suffered from poor encapsidation efficiency of the large DNA molecules by the liposomes due to a lack of DNA condensation and to charge repulsion between the lipids and nucleic acid. The encapsidation efficiency was increased somewhat by improvements in lipid processing protocols, but was significantly improved by first condensing the DNA with a polycation before adding the anionic lipid (Lee and Huang, 1996; Singhal and Huang, 1994). A second problem was their low levels of binding to the cell surface due to charge repulsion between the anionic lipids of the liposome and cell membrane. This problem could be overcome by the addition of ligands, most commonly antibodies, to the lipoplex surface to generate "immunoliposomes" that bind specific cell surface receptors (Huang *et al.*, 1984; Singhal and Huang, 1994). A third problem was that during *in vivo* delivery, both anionic and cationic liposomes could be rapidly removed from the blood stream by immune cells of the reticuloendothelial system (RES), and the addition of antibodies to the liposome surface actually enhanced this removal (Maruyama *et al.*, 1995; Singhal and Huang, 1994). RES uptake is reduced somewhat by changes in lipid composition and size, but long-circulating liposomes can also be generated by coating liposomes with polyethylene glycol (Lee and Huang, 1996; Maruyama *et al.*, 1995; Zalipsky *et al.*, 1994).

Cationic liposomes were originally designed and developed to have two advantages over the first generation of anionic liposomes (Felgner *et al.*, 1987). First, the cationic lipids readily and efficiently condense and encapsidate nucleic acids, eliminating the need for the often difficult procedures necessary to generate anionic liposomes (Farhood *et al.*, 1994; Singhal and Huang, 1994). Second, cationic liposomes need no modifications for efficient binding to the cell surface, since their positive charge mediates nonspecific adsorption to the anionic cell membrane. One disadvantage of cationic liposomes is that, because cell surface binding is ionic and therefore nonspecific, it is difficult to target gene

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delivery to specific cell types. It appears, however, that some degree of specificity can be achieved through the addition of ligands (Cheng, 1996; Kao et al., 1996). In addition, like anionic polyplexes, cationic lipids also appear to disrupt cell membranes to facilitate endosomal release (Xu and Szoka, 1996; Zelphati and Szoka, 1996). However, this release still appears to be an inefficient process (Zabner et al., 1995), leaving room for improvement in transfer efficiency through the addition of additional membrane disrupting agents to the liposomes (Budker et al., 1996). Efficiency can also be increased by improvements in lipid structure and liposome formation procedure (Singhal and Huang, 1994; Templeton et al., 1997). Finally, a detailed knowledge of lipoplex structure may lead to further enhancement (Rädler et al., 1997).

1.5.2.2 Polyplexes

Molecular conjugates, or polyplexes, are synthetic vectors generated by condensing nucleic acids with a polycation, most commonly polylysine. The polycation is often linked to a ligand to target delivery to only those cells that express a particular receptor. Since their introduction in 1987, conjugates have demonstrated a strong potential for targeted delivery, which is their most significant advantage (Wu and Wu, 1987). For example, receptor-specific delivery has been demonstrated in vitro or in vivo using a variety of ligands, including asialoglycoprotein (Wu et al., 1991; Wu and Wu, 1987), transferrin (Cotten et al., 1992; Curiel et al., 1991; Wagner et al., 1994), oligosaccharides (Monsigny et al., 1994; Perales et al., 1994), insulin (Rosenkranz et al., 1992), and epidermal growth factor (EGF) (Schaffer et al., 1997). In addition to possessing the capacity for specific delivery, conjugates can also package large DNA molecules (Cotten et al., 1992), protect their cargo from nuclease digestion (Chiou et al., 1994), and have the potential for low toxicity and immunogenicity (Plank et al., 1996; Stankovics et al., 1994). However, like lipoplexes, conjugates suffer from low efficiency. Furthermore, transgene expression is

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transient after delivery, though one group reports detectable levels of expression for up to several months (Perales *et al.*, 1994).

Several strategies have been developed to improve polyplex gene delivery efficiency. Most focus on improving the rate of endosomal release of nucleic acid into the cytoplasm, a step that was also found to be a barrier to liposome delivery as discussed above. One method to disrupt endosomal membranes borrows a technique viruses have evolved to improve efficiency. Either adenoviral virions or viral-derived fusogenic peptides are linked to polyplexes, and these agents lyse membranes at low pH to facilitate escape of DNA from endosomes (Curiel *et al.*, 1991; Plank *et al.*, 1994). Another method to disrupt endosomal membranes is to use a polycation that acts as a "proton sponge," such as polyethyleneimine or cationic dendrimers (Boussif *et al.*, 1995; Haensler and Szoka, 1993). These polymers contain a basic group with a pK_a between 4 and 8; therefore, only a fraction of the groups are protonated at neutral pH. In the acidic endosome, however, the polymer becomes increasingly protonated and buffers the endosomal pH. To overcome this buffering, the proton pumps within the endosomal membrane continue to function, leading to an increase in ionic strength and potentially the osmotic lysis of the endosome. In addition, as the polycation becomes more positively charged, ionic repulsion can lead to the disassembly of the vector, which also likely contributes to endosomal disruption.

Another means to improve conjugate efficiency focuses on the effects of conjugate structure on their biological activity. The condensation of DNA by a polycation is highly sensitive to salt concentration. Higher ionic strengths shield electrostatic interactions between the two polymers and thereby slow the complexation to lead to more selective, compact structures. Perales *et al.* used a protocol that generates uniform, compact polylysine-DNA complexes and showed that these conjugates yield higher gene transfer efficiency and sustained expression than conjugates formed by other protocols (Perales *et al.*, 1994; Perales *et al.*, 1997). Despite all these improvements, however, conjugates are still relatively inefficient.

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1.6 The Possibility of Sustained Expression

Permanent expression of a transgene after delivery is required for treating some diseases, such as inherited genetic disorders, and permanent expression requires that the transgene is either integrated into the host chromosome or is able to exist stably as an episome. In addition, if the target cell belongs to a population that is rapidly turned over, such as blood or skin cells, then the transgene must be delivered to stem cells, the self-sustaining cells that proliferate and give rise to all the short-lived cells in these tissues.

As discussed above, retroviruses and adeno-associated viruses integrate into the host chromosome. Synthetic vectors based on retrotransposons are third type of integrating construct with potential for sustained delivery (Hodgson et al., 1996). These vectors have the potential to yield transgene expression for the life of the target cell and all its progeny, if problems with transcriptional silencing of the transgene over time can be solved (Neff et al., 1997; Qin et al., 1997).

The alternative to integration is vectors which can be maintained episomally after delivery. However, if the target cell is dividing, the vector must also be able to replicate extrachromosomally to prevent dilution of the transgene in the proliferating cell population. As discussed above, proteins encoded in the adenoviral genome may allow the stable, extrachromosomal maintenance of the viral genome, if elimination of transduced cells by the immune system can be avoided (Lieber et al., 1997). In addition, vectors that borrow the EBNA-1 protein and oriP element from the Epstein Barr virus have the potential for episomal maintenance (Calos, 1996). Finally, artificial human minichromosomes that would stably replicate along with the host chromosomes are under development; however, delivery of these huge constructs remains a problem (Harrington et al., 1997).

1.7 Gene Therapy: Current Status

Gene therapy has tremendous therapeutic potential, but currently it remains just that: potential. In the 19 years since the first delivery of a transgene to a mammalian cell

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(Mulligan *et al.*, 1979), there has been substantial progress in the development of viral and synthetic vectors, most of which can now efficiently deliver genes *in vitro*. However, gene delivery technology still needs vast improvement before efficient delivery and sustained expression become possible *in vivo*.

One lesson that has emerged from gene delivery research is that there will likely not be a single vector that is perfectly suited for all applications. That is, each vector has strengths that make it appropriate for certain applications, if its weaknesses can be overcome. For example, the capacity for relatively efficient *ex vivo* delivery and chromosomal integration may make retroviral vectors suitable for gene transfer to hematopoietic stem cells (Kaptein *et al.*, 1997; Nolta *et al.*, 1996). In contrast, adenoviral vectors may be used for *in vivo* delivery to the liver and lung, but currently they cannot efficiently deliver to hematopoietic derived cells (Wilson, 1996; Wilson, 1996). Synthetic vectors have tremendous potential for targeted delivery to a number of systems with little toxicity or immunogenicity, but the efficiency and stability of expression must be improved. For now, synthetic vectors can be used in applications where low efficiency and transient expression are sufficient, such as in DNA vaccines.

There have been over 200 clinical trials initiated in the United States and over 30 internationally since the first in 1990 (Rosenberg *et al.*, 1997). A number of trials have demonstrated that gene therapy can be a safe procedure and that genes can be delivered and at least transiently expressed. However, efficacy has been extremely limited (Crystal, 1995; Ross *et al.*, 1996; Verma and Somia, 1997). Permanent cures of inherited genetic disorders such as ADA deficiency or cystic fibrosis are far beyond the capability of current vectors with low efficiency and unstable expression, though transient transgene expression has been achieved in several trials and low level sustained expression of ADA in several patients (McElvaney, 1996; McLachlan *et al.*, 1996; Onodera *et al.*, 1998). In the shorter term, trials for cancer, which account for approximately half of all trials, may encounter more success since the necessary duration of transgene expression is shorter and at most is

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required until the malignancy is eliminated. There have in fact been sporadic reports of clinical responses to immunotherapy approaches to cancer treatment (Abdel-Wahab *et al.*, 1997; Ross *et al.*, 1996). However, successes in any clinical trials will continue to be extremely low until the vectors they rely upon are substantially improved.
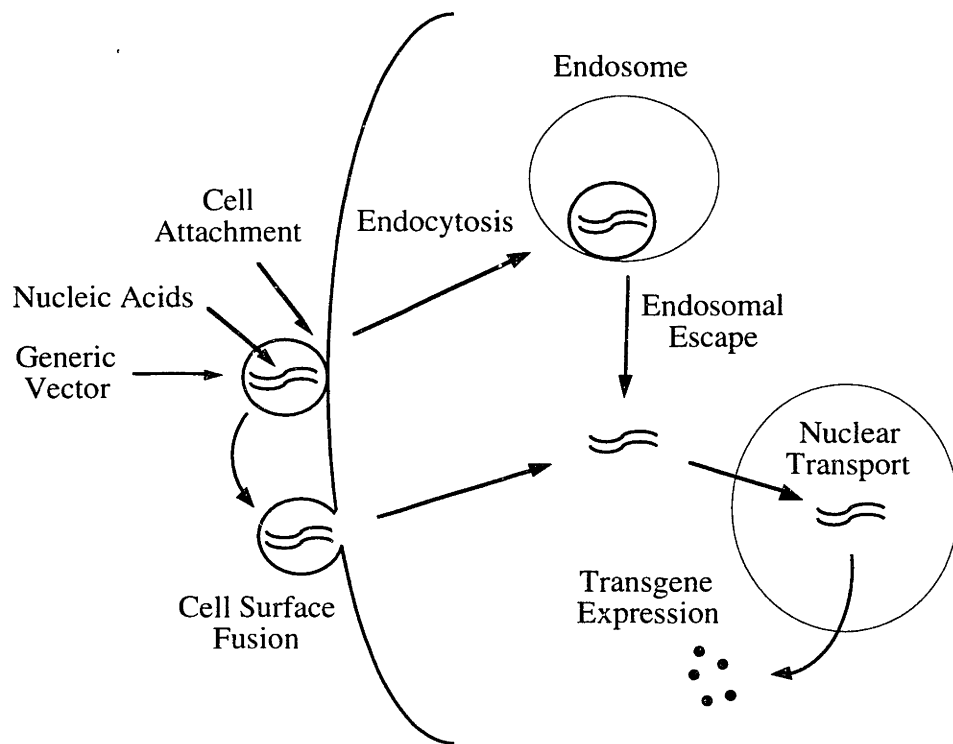
Figure 1.1 Pathways of vector gene delivery.

"Natural" Vectors:

retrovirus
adenovirus/AAV
herpes virus

- replication
+ transgene

viral vector

- viral
genes

Synthetic "Virus" ?

"Synthetic" Vectors:

targeted
liposomes

"fusion"
polyplexes

+ ligand

liposomes

+ lipid

+ membrane
disrupting
agent

targeted
polyplex

naked DNA

+ polycation   polyplex   + ligand

Figure 1.2 "Evolution" of viral and synthetic gene delivery vectors

1.8 References

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2. Experimental System

2.1 Background

Molecular conjugates, or polyplexes, are vectors with substantial promise for targeted, *in vivo* gene delivery; however, they are currently limited by low gene delivery efficiency. We believe that gaining mechanistic information on the cell biology of molecular conjugate gene delivery can yield further improvements in transfer efficiency by revealing barriers to conjugate transfer from the cell surface to the nucleus. To reach this goal, we have developed an experimental system that employs epidermal growth factor (EGF) as the ligand to direct delivery of DNA encoding the green fluorescent protein (GFP) to mouse fibroblasts. This system is designed to build upon the extensive, basic understanding of the cell biology of the EGF receptor-ligand system gained over several decades. In addition, to crosslink EGF to the conjugates we use a streptavidin-biotin crosslinking chemistry that allows significant modularity in tethering ligands or peptides to polylysine. Finally, we employ the green fluorescent protein as the reporter gene because its expression can be readily visualized or quantified by relatively simple assays. After developing this system, we will employ it to identify rate-limiting steps in gene delivery and will improve conjugates to overcome these limitations.

2.1.1 EGF and its Receptor

Receptors are important components of the mechanisms by which cells recognize their environment, respond to external stimuli or signals, and selectively import certain nutrient molecules. Because the repertoire of receptors a cell expresses fundamentally modulates or controls cell behavior, the presence of specific receptors is critical in determining the specificity and selectivity of cell function (Wiley, 1992). Therefore, receptors are often the targets of medical intervention designed to elicit a desired response within a target cell population (Hodgson, 1992).

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Growth factors are soluble peptide signal molecules that act upon cells by binding to specific cell surface receptors. In 1960, Stanley Cohen discovered the first growth factor, epidermal growth factor, and showed that it accelerated eyelid opening and tooth eruption in newborn mice (Cohen, 1962). Later characterization of the gene and protein revealed that EGF is synthesized as a 1207 amino acid, transmembrane precursor that is cleaved into the 53 amino acid secreted form, which is shown in Figure 2.1 (Bell *et al.*, 1986; Scott *et al.*, 1983). Since its isolation, EGF has been found to be only one of a number of proteins in the epidermal growth factor family of ligands that bind the EGF receptor (EGFR), which also includes TGFα and amphiregulin among others (Carpenter and Wahl, 1990).

The epidermal growth factor receptor (EGFR), shown in Figure 2.2, is a 170 kDa transmembrane glycoprotein composed of four domains: the extracellular, transmembrane, juxtamembrane, and cytoplasmic. The extracellular domain contains the ligand binding site, while the cytoplasmic region includes the tyrosine kinase domain and a regulatory region. The binding of a ligand activates the EGFR tyrosine kinase activity, initiating an intracellular signal transduction cascade leading to changes in gene transcription (van der Geer, 1994). Ultimately, EGFR activation regulates cell proliferation, migration, or differentiation in a variety of tissues *in vivo*, including bone, the immune system, muscle, the nervous system, and the vascular system (Carpenter and Wahl, 1990).

In order to modulate the magnitude and duration of a ligand-induced signal, cells have developed a number of mechanisms to downregulate the number and activity of cell surface receptors. For example, receptor-ligand complexes can be removed from the cell surface by receptor-mediated endocytosis and subsequently degraded, as shown in Figure 2.3. Upon receptor binding and kinase activation, a growth factor receptor-ligand complex is specifically recruited to coated pits, specialized regions of the cell surface. After the membrane within these pits invaginates and is endocytosed, the resulting vesicle fuses with the sorting endosome. Within this endosome, a trafficking process can route the receptor-

ligand complex to the lysosome for degradation, or recycle it to the cell surface (Trowbridge *et al.*, 1993; Wiley, 1992). In addition, the receptor and ligand may dissociate to a certain extent in the low pH environment of the endosome, so that the cell can add another degree of complexity to the trafficking process by sorting them together or separately (French *et al.*, 1995; Goldstein *et al.*, 1985; Hopkins and Trowbridge, 1983).

The use of the EGF as a model growth factor system has revealed important phenomena of growth factor and receptor trafficking through a cell. Specifically, varying the characteristics of the receptor and ligands, through the use of a large number of receptor and ligand mutants, has led to the identification of important molecular properties of these proteins that govern their rates of transport through, and therefore their distributions within, the various compartments of the endosomal network (Chang *et al.*, 1993; French *et al.*, 1994; French *et al.*, 1995; Herbst *et al.*, 1994; Opresko *et al.*, 1995).

There is a growing body of literature that maintains that after endocytosis, growth factors and/or their receptors are sorted to the nucleus in addition to being recycled or degraded (Burwen and Jones, 1987; Rakowicz-Szulczynska, 1994). There is evidence for the nuclear localization of EGF (Cao *et al.*, 1995; Holt *et al.*, 1995; Johnson *et al.*, 1980; Murawsky *et al.*, 1990; Rakowicz-Szulczynska *et al.*, 1989), NGF (Andres *et al.*, 1977; Rakowicz-Szulczynska, 1993; Yanker and Shooter, 1979), PDGF (Rakowicz-Szulczynska *et al.*, 1986; van den Eijnden-van Raaij *et al.*, 1988), FGF (Baldin *et al.*, 1990; Bouche *et al.*, 1987; Tessler and Neufeld, 1990), and insulin (Smith and Jarett, 1987), among others. Although the findings are controversial and considered by some to be artifactual (Evans and Bergeron, 1988), a number of investigators have detected nuclear localization in a variety of cell systems by a variety of techniques, and have found evidence for several physiological functions of such localization.

EGF is a highly suitable choice as a ligand for receptor-mediated gene delivery for several reasons. First, as reviewed above, the EGF system is the best characterized receptor-ligand system in cell biology and therefore provides a substantial knowledge base

upon which fundamental studies of gene delivery can be built. In addition, it is advantageous as an experimental system, since a number of receptor and ligand mutants are available if we choose to study the effects of receptor-ligand dynamics on gene delivery. Furthermore, although EGFR itself is not selectively expressed by specific cell populations, other growth factor receptors are, particularly in the immune and nervous systems (Bothwell, 1991; Kordower *et al.*, 1993; Nicola, 1994). Therefore, EGFR-mediated delivery is a good model for targeted delivery to growth factors *in vivo*. Finally, there is substantial evidence of growth factor receptor trafficking to the cell nucleus, so EGFR may provide a conduit for transport of conjugates from the surface all the way to the nucleus.

2.1.2 Crosslinking Chemistry

The majority of molecular conjugate systems use a ligand that is directly crosslinked to polylysine; however, we tether EGF to the conjugate by using a biotin-streptavidin bridge. We first crosslink streptavidin to polylysine and then biotinylate EGF. This approach has the major advantage that, by incorporating streptavidin into the conjugates, we can then use the high affinity between streptavidin and biotin to easily add variable amounts of any ligand or peptide to the polyplexes. This high degree of modularity will aid future experiments. A further advantage is that biotinylation is a simple and high-yield reaction, leading to little loss of costly ligands like growth factors.

2.1.3 Green Fluorescent Protein

GFP is a 27 kDa protein produced by the bioluminescent jellyfish *Aequorea victoria*. The protein becomes florescent shortly after synthesis due to the cyclization of three amino acids to generate a chromophore, and this reaction does not involve any cofactors unique to *A. victoria*. Since Chalfie *et al.* first reported its use as a marker for gene expression, there has been an enormous upsurge in both interest and potential applications for GFP, aided by the development of a number of GFP mutants that are

brighter than or have different spectral properties from the wild type protein (Chalfie *et al.*, 1994; Cormack *et al.*, 1996; Crameri *et al.*, 1996; Cubitt *et al.*, 1995; Heim *et al.*, 1995; Zolotukhin *et al.*, 1996). As a reporter gene to quantify gene expression after delivery, GFP has advantages over traditional reporters such as chloramphenicol acetyl transferase (CAT), β-galactosidase, and luciferase. These reporters require enzymatic reaction steps to quantify expression. In contrast, GFP fluorescence can be detected directly without any reaction steps by using fluorescence activated cell sorting (FACS) or fluorescence microscopy analysis. We use a GFP gene expressed from the strong human cytomegalovirus promoter. Additionally, the gene contains a serine to threonine mutation at position 65 that significantly increase brightness, as well as numerous silent mutations to optimize codon usage for mammalian expression.

2.1.4 Conceptual Approach for Improving Gene Delivery Efficiency

By condensing plasmid DNA encoding GFP with polylysine linked to streptavidin, and then tethering EGF to the complex to generate a molecular conjugate, we can generate conjugates that bind to the EGFR. As shown in Figure 2.4, these ligands mediate the binding of the conjugate to a specific cell surface receptor, in this case the EGFR. The receptor is then internalized, and the conjugate is thereby conveyed by the receptor-ligand pair to the inside of the cell. Once inside the cell, a small fraction of the conjugates is able to escape the endosome to the cytoplasm. Finally, a fraction of these translocates to the nucleus, where genes contained in the conjugate DNA are expressed. We are conceptualizing the passage of the molecular conjugate from the cell surface to the nucleus as a series of potentially rate-limiting steps, or barriers, to gene delivery. These steps can include cell surface binding, internalization, endosomal escape, nuclear transport, and RNA transcription from the conjugate DNA. After developing our experimental system, we conduct experiments that demonstrate that the first and last steps, cell surface binding and

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RNA transcription, are rate-limiting steps. Furthermore, we engineer the conjugates to overcome these barriers and thereby significantly improve delivery efficiency.

2.2 Materials and Methods

2.2.1 Materials

Mouse EGF, penicillin-streptomycin, L-glutamine, and trypsin-EDTA were obtained from Life Technologies (Gaithersburg, MD). Acrylamide, TEMED, and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories, and high range molecular weight standards from Amersham Life Science. Mouse EGF was iodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) using Iodobeads and following the manufacturer's protocol (Pierce Chemicals, Rockford, IL). Iodinated protein was separated from free iodine by gel filtration with G-15 (Pharmacia Biotech, Piscataway, NJ), and the specific activity ranged from 160,000 to 240,000 cpm/ng. Unless otherwise mentioned, all other reagents were obtained from Sigma Chemicals (St. Louis, MO).

2.2.2 Preparation of GFP Plasmid DNA

The plasmid phGFP-S65T, a kind gift of Brian Seed (Harvard Medical School), contains a gene with optimized human codon usage encoding the green fluorescent protein serine-65 to threonine mutant under the control of the human cytomegalovirus immediate early promoter. The plasmid was propagated in the MC1061/P3 E. Coli strain and purified with a EndoFree Plasmid Maxi Kit (Qiagen).

2.2.3 Streptavidin-Polylysine Crosslinking and Purification

One quarter μmol of poly-L-lysine (chain length 182) was reacted with 0.75 μmoles of the heterobifunctional reagent SPDP in 0.5 ml of HBS (20 mM HEPES, pH 7.0, 150 mM NaCl) for 2 h at room temperature. The polylysine solution was dialyzed in 2000 MWCO tubing (Spectrum, Houston, TX) twice against 1 l of HBS. Five μl of 1 M TCEP

(Pierce) were added to the polylysine solution, and it was incubated at room temperature under nitrogen for 10 min in order to reduce the disulfide groups of the SPDP residues. The number of free sulfhydryls per polylysine chain was determined by quantifying the amount of 2-mercaptopyridine in the resulting solution by measuring its absorbance at 340 nm using a Spectromax 250 spectrophotometer (Molecular Devices). Polylysine was quantified by the OPA Protein Assay (Pierce) with a RF-5000 Spectrofluorophotometer (Shimadzu) using pure polylysine as a standard. Six nmol of polylysine with free sulfhydryl groups were reacted with 0.2 mg (3.3 nmol) of streptavidin-maleimide in HBS for 18 h at room temperature.

The crude reaction mixture was diluted into 1 ml of carbonate buffer (50 mM sodium carbonate, 0.5 M NaCl, pH 11) and loaded onto a column containing 1 ml of 2-iminobiotin-agarose. The column was washed with 5 ml of the carbonate buffer and eluted with 0.1 N acetic acid. The eluant was diluted to 3 ml with a buffer containing 50 mM HEPES and 0.5 M NaCl (pH 7.0) and loaded onto a HiTrap SP column (Pharmacia Biotech). The column was washed with 5 ml of the 0.5 M salt solution and eluted with a buffer containing 50 mM HEPES and 2 M NaCl (pH 7.0). Streptavidin was quantified in the 0.25 ml fractions collected from both columns by checking the absorbance at 280 nm using the Spectromax 250 spectrophotometer.

The reaction and purification products were analyzed by SDS-polyacrylamide gel electrophoresis in a 10% gel using an XCell II Mini-Cell apparatus (Novex Electrophoresis), and gels were stained with Coomassie Blue R-250 (Life Technologies). The DNA binding activity of purified streptavidin-polylysine was assessed by agarose gel electrophoresis using a Minigel Electrophoresis System (Owl Scientific). Briefly, varying amounts of streptavidin-polylysine were added to 0.5 μg of GFP plasmid in 10 mM HEPES (pH 7.0) and 1 M NaCl, and the complexes were analyzed on a 1% agarose gel using a Tris-acetic acid buffer system.

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2.2.4 EGF Biotinylation

Sulfo-NHS-LC-LC-Biotin (Pierce) was dissolved in HBS at 10 mg/ml, and added to 50 μl of 1 mg/ml mouse EGF in HBS. The solution was agitated for 4 h at room temperature, and the excess biotinylation reagent was removed from EGF by gel filtration on an 4 ml column of G-15 (Pharmacia Biotech). EGF was quantified by absorbance at 280 nm.

2.2.5 Cell Culture

B82 L mouse fibroblast cells, which lack endogenous EGF receptors and were previously transfected with the wild type human EGF receptor and selected with methotrexate, were a generous gift of G.N. Gill (University of California at San Diego). Transfected cells were maintained with 1 μM methotrexate and grown in DME medium supplemented with 10% dialyzed calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 2.5 μg/ml streptomycin at 37°C in a humidified atmosphere of air and 5% CO_2.

2.2.6 Competition Binding Assay

The ability of EGF-biotin-streptavidin-polylysine to bind to the EGF receptor was assessed by a radioactive competition binding experiment. B82 cells were grown to confluence on 35 mm dishes (Corning) and were switched to DME with no bicarbonate containing 20 mM HEPES (pH 7.4) and 1 mg/ml bovine serum albumin (D/H/B) 2 h before experiments. From 0.2 to 20 pmol of biotinylated EGF were added to 40 pmol of streptavidin crosslinked to polylysine in 0.5 ml D/H/B and incubated for 1 h at room temperature. These solutions were then mixed with radiolabeled EGF such that the final concentrations were 1 nM ^{125}I-EGF and 0.1-10 nM of biotinylated EGF with streptavidin-polylysine. The mixtures were incubated with cells in duplicate samples for 3 h at 4°C, and the cells were then rinsed three times with 3 ml of ice-cold WHIPS buffer (20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mg/ml

polyvinylpyrrolidone). The cells were solubilized with 1 N NaOH, and the cell-bound ^{125}I-EGF counted with a Packard 5000 series γ-counter.

2.2.7 Receptor-Mediated Gene Delivery

To 20 μl of HBS (20 mM HEPES, pH 7.4, 150 mM NaCl) were added 4.9 pmoles of biotinylated EGF and 4.9 pmoles of streptavidin linked to polylysine. After 15 min at room temperature, the EGF-biotin-streptavidin-polylysine was mixed with 1 μg of phGFP-S65T plasmid in 80 μl of HBS. The solution was added to 1 ml of OptiMem Reduced Serum Media (Life Technologies), and 100 mM chloroquine and 1 mg/ml EGF were added to some samples to concentrations of 100 μM and 2 μg/ml, respectively. B82 cells were seeded at 5 x 10^4 cells/cm^2 in 35 mm dishes (Corning), grown for 12 h, and switched to D/H/B 2 h before experiments. The OptiMem solutions were added to cells for 4 h and was then aspirated and replaced with growth media. After 24 h, the cells were trypsinized and analyzed for expression of GFP using a FACScan flow cytometer (Becton Dickson) with a 488 nm excitation laser and a 530/30 nm fluorescein emission filter.

2.3 Results and Discussion

Reaction of polylysine with a threefold molar excess of SPDP followed by reduction with TCEP resulted in the introduction of 2.8 free sulfhydryls per polymer chain, as quantified by the absorption at 340 nm of the 2-mercaptopyridine released from SPDP residues upon reduction (data not shown). Excess polylysine-sulfhydryl was reacted with streptavidin-maleimide, and the crude reaction mixture was loaded onto a 2-iminobiotin column at pH 11. Active streptavidin bound to the column at high pH and was eluted with an acidic solution (Figure 2.5a). The eluant was loaded onto a cation exchange column and eluted with 2 M salt (Figure 2.5b). This novel combination of affinity and cation exchange chromatography should yield pure crosslinked streptavidin-polylysine.

The crosslinking and successful purification were confirmed by SDS-PAGE (Figure 2.6). As shown by the 60 kDa band in lane 2, the addition of SDS alone to streptavidin does not separate the tetramer into its subunits. However, boiling the protein in SDS prior to loading dissociates streptavidin into its 15 kDa monomers (lane 3). The absence of any bands in lane 8 is due to the fact that SDS precipitates polylysine, as well as any protein linked to polylysine, so that these species cannot migrate into the gel. The appearance of faint 60 and 15 kDa bands in the unboiled crude reaction mixture (lane 4) indicates that only a trace amount of streptavidin is unlinked to polylysine. Since it is unlikely that each monomer of a polylysine-crosslinked tetramer is bound to a polylysine chain, boiling of crosslinked proteins should liberate unlinked monomers. This explains the appearance of intense bands in the boiled crude reaction solution (lane 5). The ladder of bands of molecular weight multiples of 15 kDa in this lane corresponds to oligomers of streptavidin subunits. The absence of protein in lane 6 indicates a successful purification, since there is no streptavidin unlinked to polylysine in the purified product. However, boiling of this product liberates the monomers not crosslinked to polylysine, which appear as a single, 15 kDa band (lane 7). Quantification of streptavidin and polylysine in the purified product indicates that there are 3.5 polylysine chains per streptavidin molecule (data not shown).

The DNA binding activity of purified streptavidin-polylysine was demonstrated by its ability to retard the migration of DNA in agarose gel electrophoresis (Figure 2.7). Lane 2 shows uncut plasmid. A large molar excess of streptavidin does not bind the DNA (lane 3), while excess pure polylysine completely retards the migration of the GFP plasmid in the gel (lane 4). Lanes 5 through 9 show the migration of plasmid samples containing decreasing amounts of streptavidin-polylysine, or a decreasing ratio of positively-charged lysine residues per negatively-charged phosphate of the DNA. At a positive to negative charge ratio of 1 (lane 7), the streptavidin-polylysine binds the DNA such that the electroneutral complex does not migrate. In contrast, the DNA is only partially retarded at a

ratio of 0.1 (lane 9), and at a positive to negative charge ratio of 10, the DNA does not enter the gel (lane 5).

Mouse EGF was biotinylated on its only amino residue, the N-terminus, using the reagent Sulfo-NHS-LC-LC-Biotin. Biotinylated EGF, both in the absence and presence of streptavidin-polylysine, can bind to the EGF receptor on B82 fibroblasts. As shown in Figure 2.8, the abilities of varying concentrations of EGF and biotin-EGF to displace the binding of a constant concentration of radiolabeled EGF were similar, which indicates that the linkage of biotin to EGF does not affect its affinity for its receptor on B82 cells. EGF-biotin-streptavidin-polylysine does show a slight shift in its binding profile; however, it still retains the ability to bind to the receptor with high affinity. Streptavidin-polylysine alone does not inhibit the binding of ^{125}I-EGF to the EGFR (data not shown).

To demonstrate receptor-mediated gene delivery, biotinylated EGF was incubated with streptavidin-polylysine, and the phGFP-S65T plasmid was added at an electroneutral polylysine to DNA ratio to generate conjugates schematically shown in Figure 2.9. The molecular conjugate was incubated with cells for 4 h, aspirated, and replaced with regular growth media. Chloroquine (100 µM) and EGF (2 µg/ml) were added with the conjugates to some samples. After 24 h, the cells were trypsinized, suspended, and analyzed for expression of GFP by FACS, as shown in Figure 2.10. Approximately 0.46% of the cells incubated with EGF molecular conjugate and chloroquine were fluorescent. The two negative controls, which yielded no cells expressing GFP, were samples incubated in chloroquine either in the absence of conjugate or in the presence of conjugate plus 2 µg/ml (0.33 µM) free mouse EGF. This high concentration of free ligand saturates the EGF receptors and thereby prevents conjugate from binding to and entering the fibroblasts. The average level of fluorescence of cells expressing GFP was 160 fluorescence units, as compared to 5.6 in the negative controls. The transfection efficiency of EGF conjugates in B82 cells is comparable to that of both the calcium phosphate method and lipofection, using the reagent LipofectAMINE (Life Technologies) (data not shown). We did not observe

fluorescent cells in the absence of chloroquine, a compound that inhibits endosomal acidification and lysosomal degradation. Although this compound is widely used as an antimalarial agent and could therefore potentially be employed *in vivo* to enhance gene delivery, it would be preferable to improve the conjugates so that they did not depend on chloroquine for delivery. Finally, we did not observe fluorescence in cells transfected with a similar plasmid encoding wild type GFP (data not shown). However, even in the absence of an enzymatic amplification of signal, the mutant GFP is an amply sensitive reporter gene for this application.

This gene delivery system is well suited for investigations of the mechanism of gene delivery for several reasons. We can build upon the knowledge of the EGF system to study the effects of receptor-ligand dynamics in gene delivery. In addition, the modular biotin-streptavidin linkage can readily allow the incorporation of any ligands or peptides into the system. Finally, this first use of GFP as a reporter gene for molecular conjugate gene delivery allows rapid imaging and quantitation of gene expression. Now that we have developed a functional receptor-mediated gene delivery system, we can proceed with experiments to identify rate-limiting steps in gene delivery and engineer the conjugates to overcome them in order to improve gene delivery efficiency.

a)
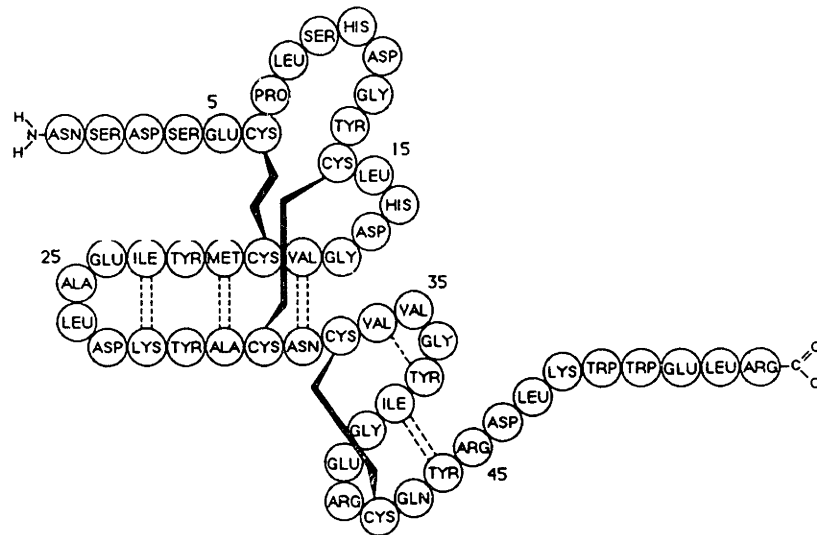
b)

```
        1         10        20        30        40        50
        |         |         |         |         |         |

mouse EGF   NSYPGCPSSYDGYCLNGGVCMHIESLDSYTCNCVIGYSGDRCQTRDLRWWELR

human EGF   NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR

TGFα        VVSHFNDCPDSHTQFCFHGTCRFLVQEDKPACVCHSGYVGARCEHADLLA
```

Figure 2.1 a) β-sheet structure of human EGF, and b) sequences of EGF family members.

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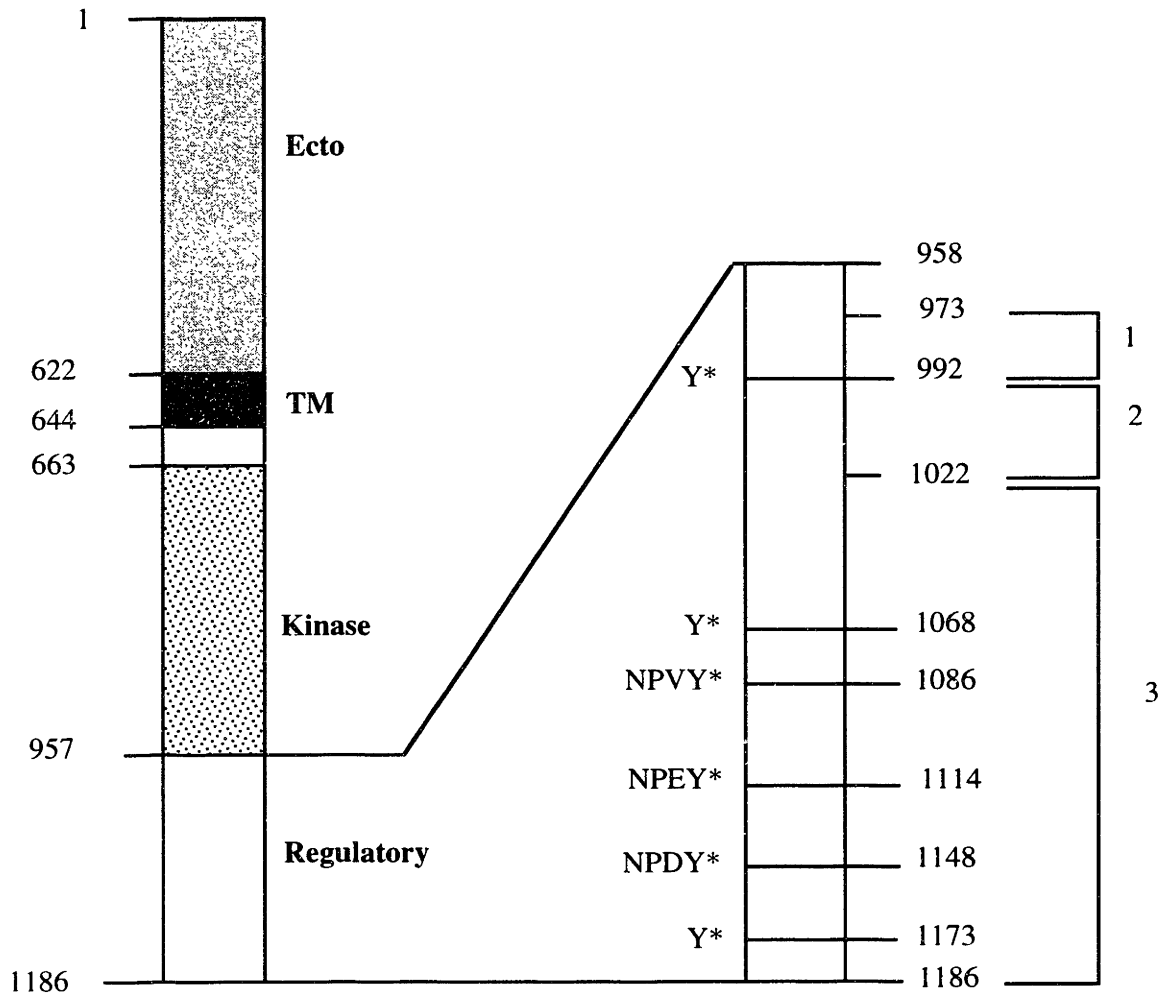
Figure 2.2 EGF receptor domains

Binding

Ligand

Receptor

Endocytosis

Recycling

Sorting
Endosome

Degradation

Nuclear
Transport?

Lysosome

Nucleus
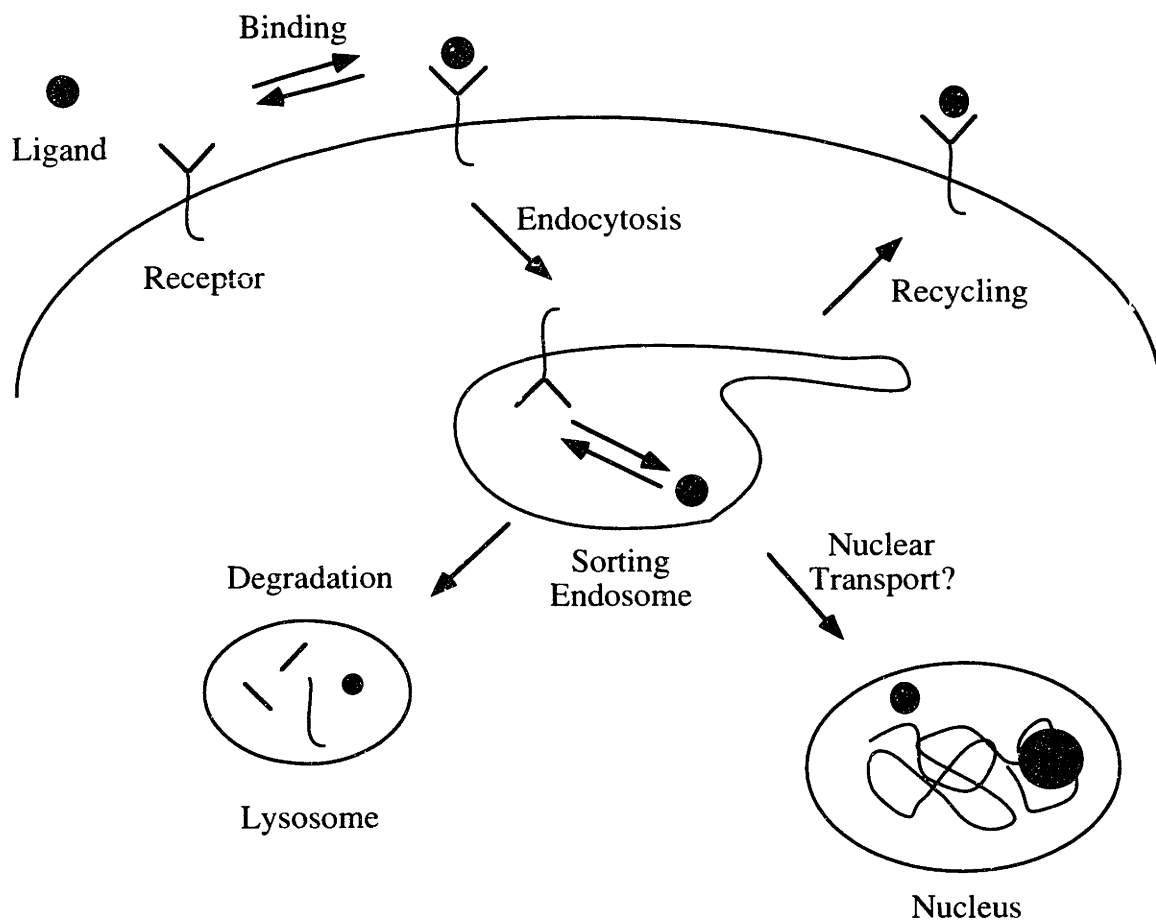
Figure 2.3 Receptor-ligand trafficking pathways

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1. Surface
   Binding

Molecular
Conjugate

2. Internalization

Recycling

3. Endosomal
   Escape

4. Nuclear
   Translocation

Lysosomal
Degradation

5. RNA
   Transcription
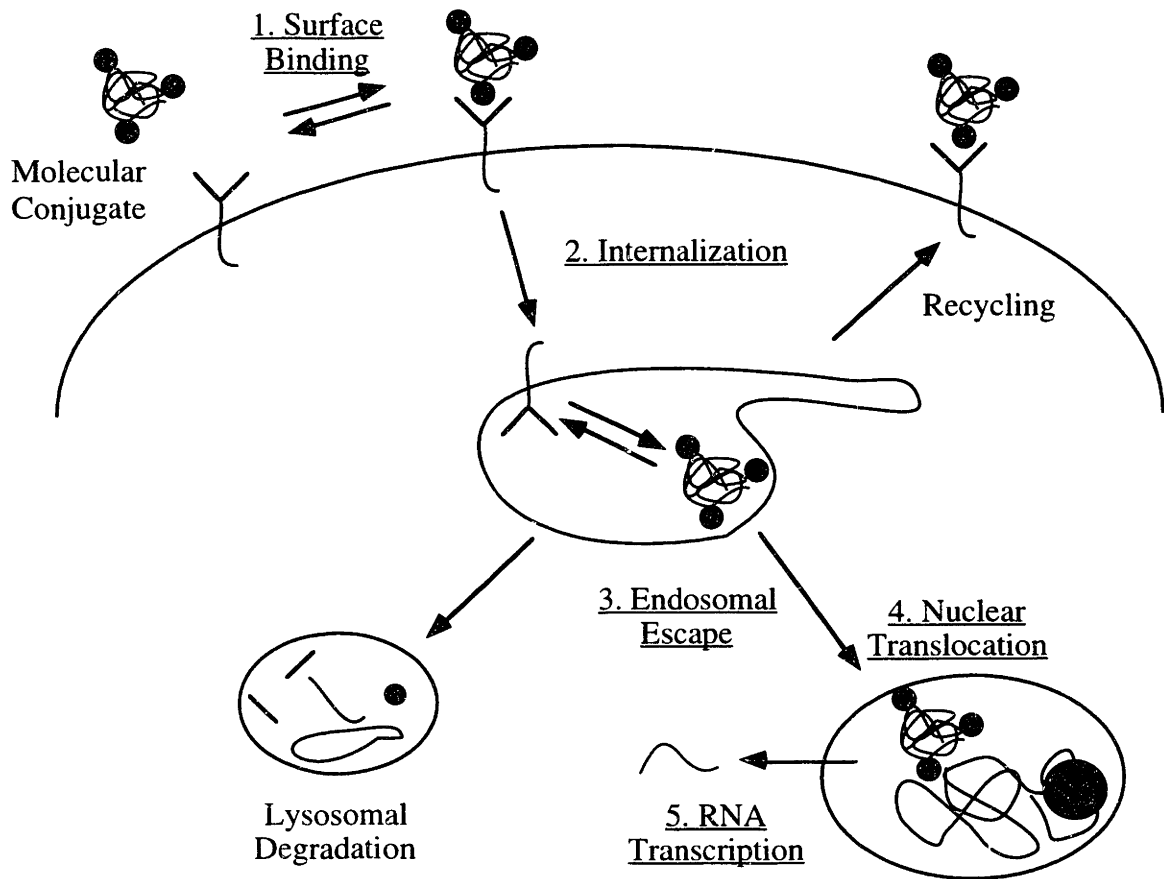
Figure 2.4 Molecular conjugate delivery pathway
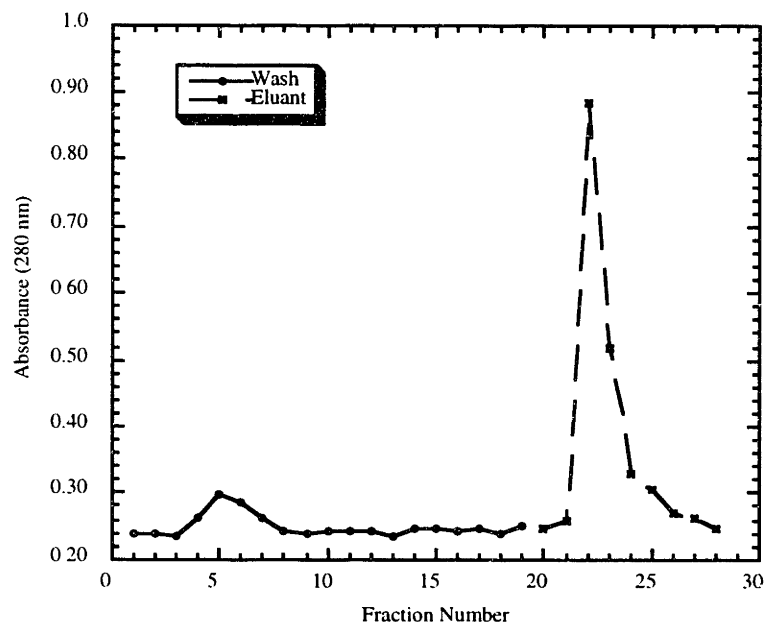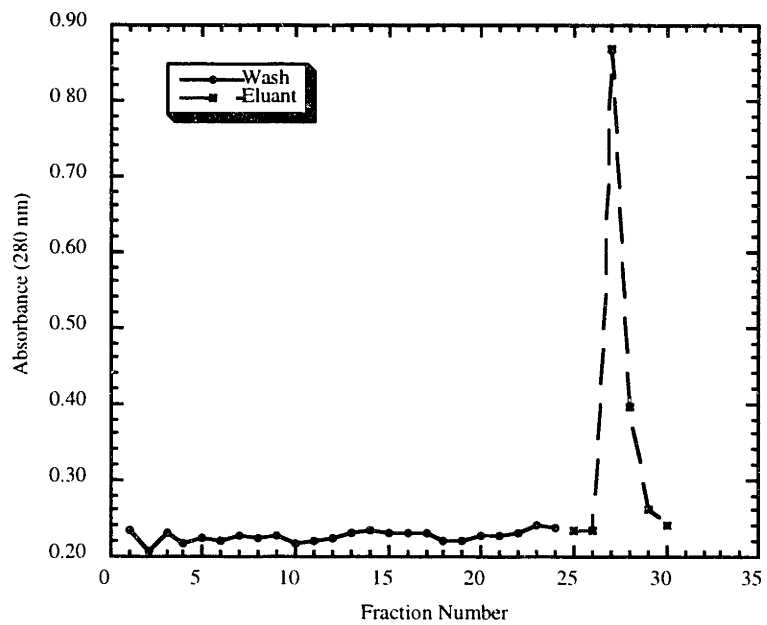
Figure 2.5 Streptavidin-polylysine purification with sequential a) 2-iminobiotin and b) cation exchange columns

60 kDa

15 kDa

1 2 3 4 5 6 7 8
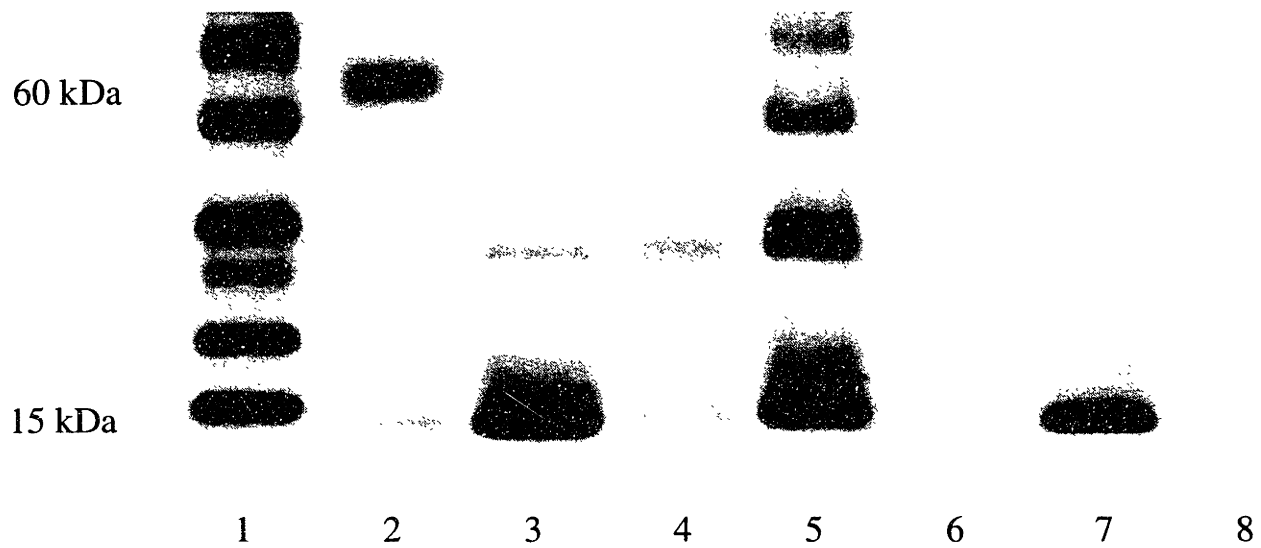
Figure 2.6 SDS-PAGE analysis of streptavidin-polylysine crosslinking and purification

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plasmid ⌐

pure streptavidin ⌐

pure polylysine ⌐

10   2   1   0.5   0.1

lysine/nucleotide
ratio
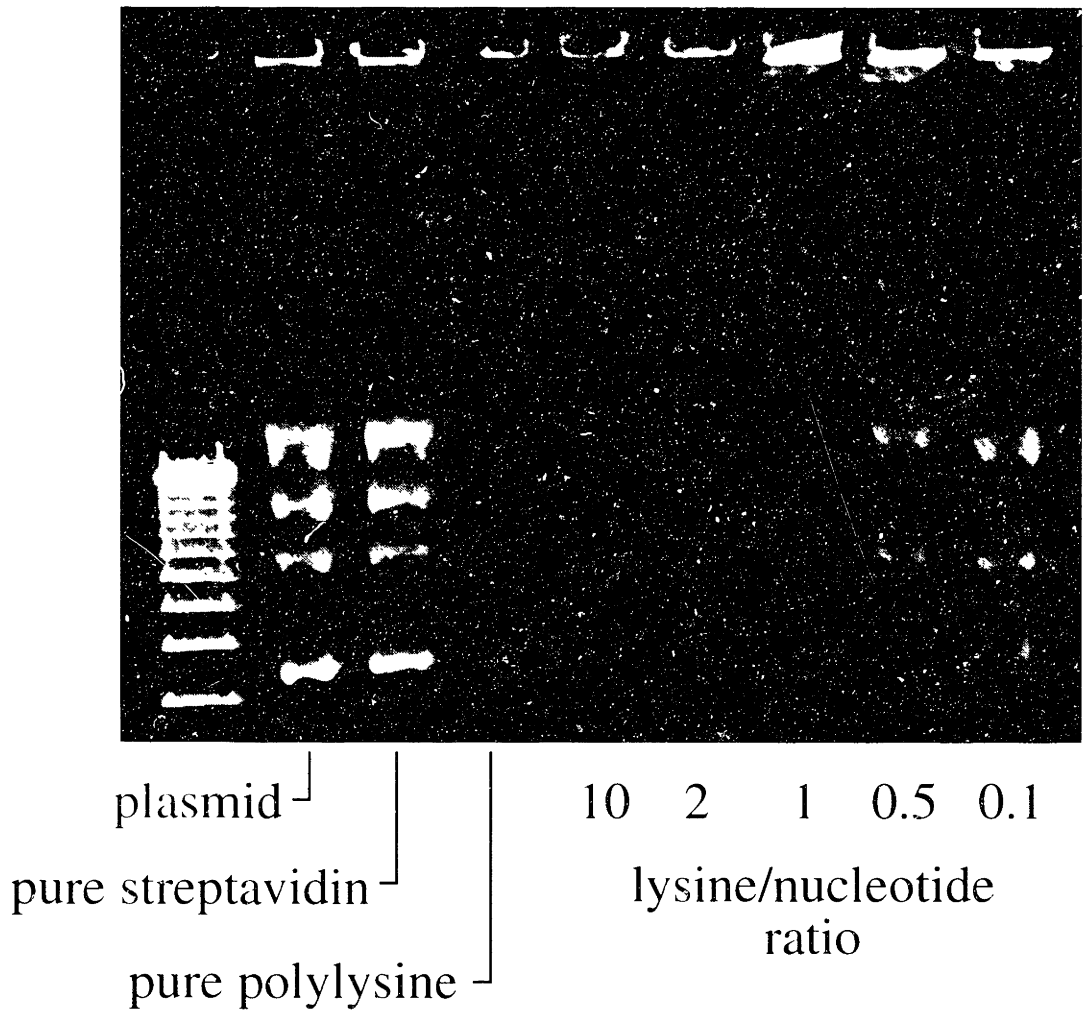
Figure 2.7 Agarose gel demonstration of streptavidin-polylysine binding to DNA
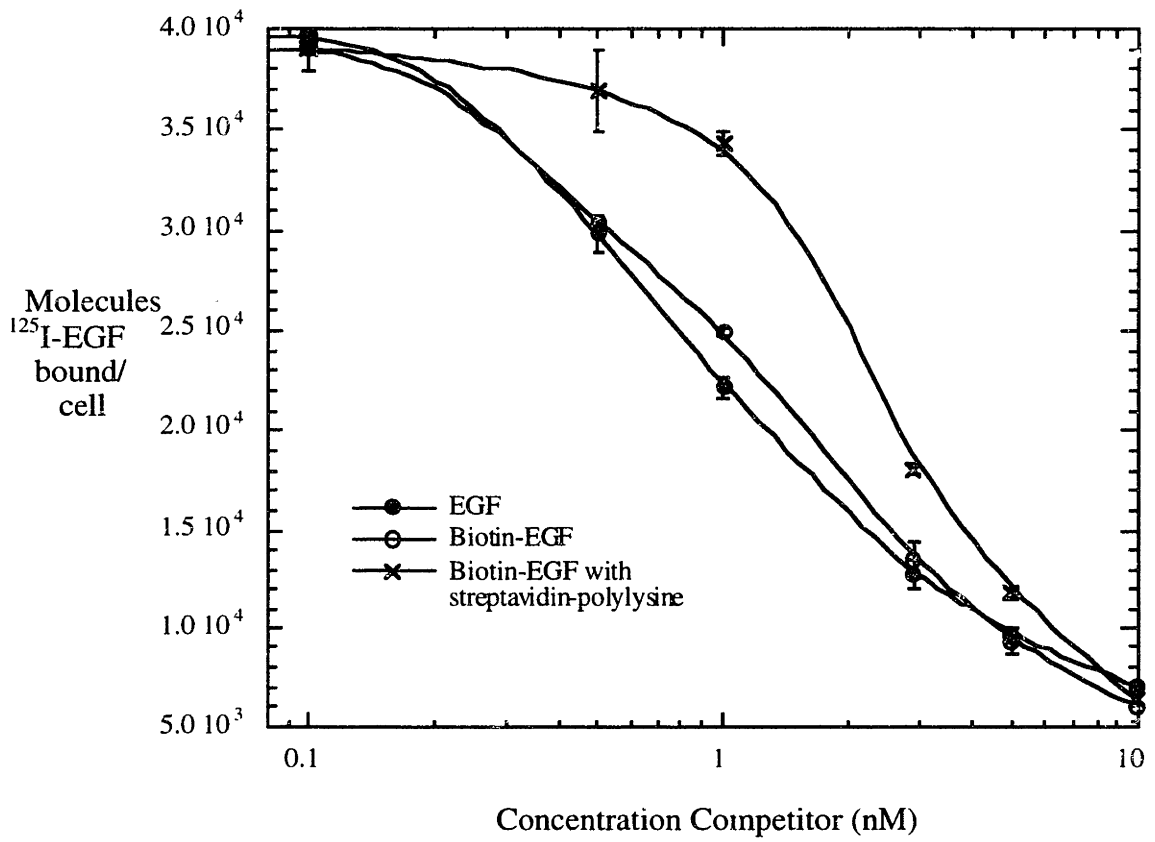
Figure 2.8 Binding of biotin-EGF to EGFR

Streptavidin

Biotin

GFP Plasmid   **Polylysine**   **EGF**
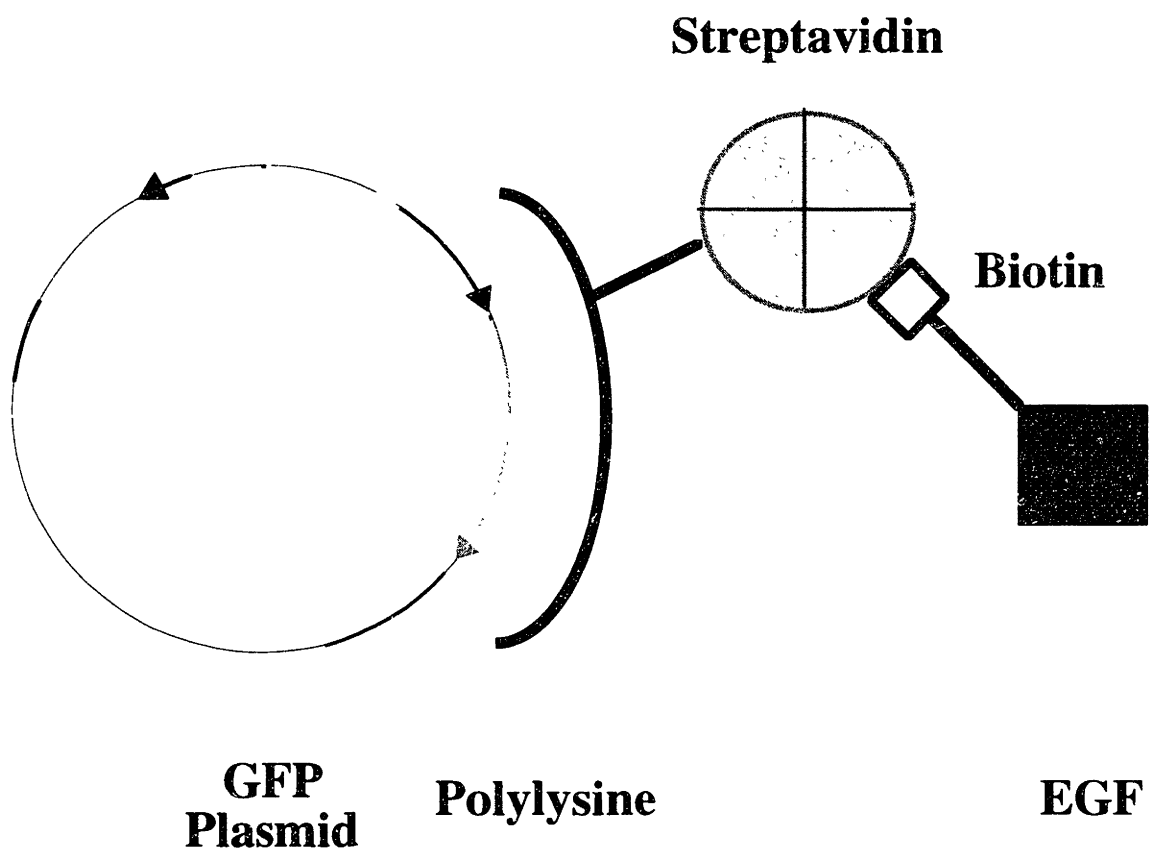
Figure 2.9 Schematic of molecular conjugate structure
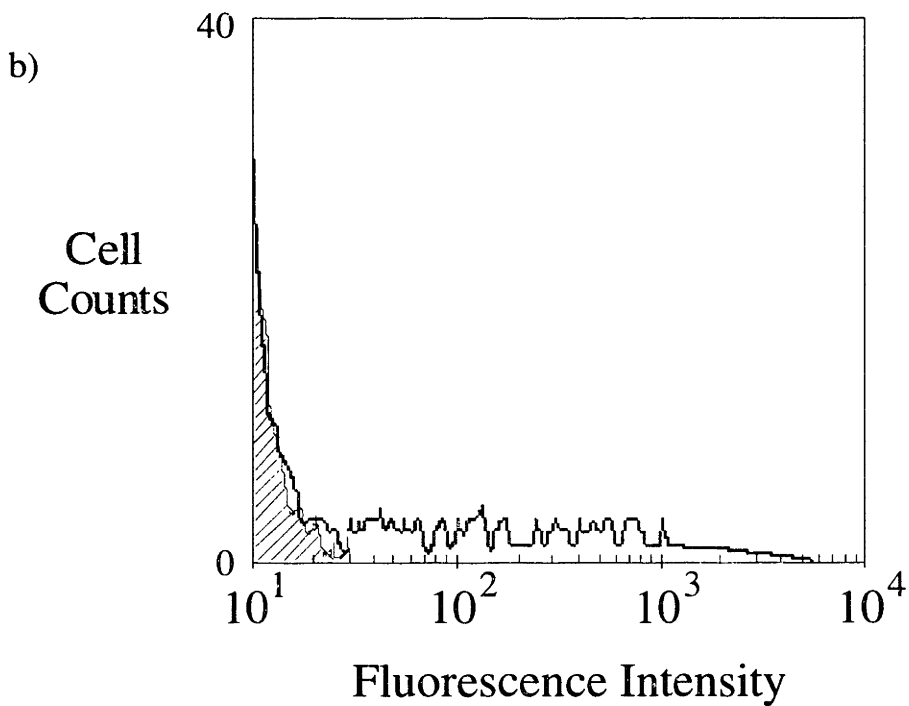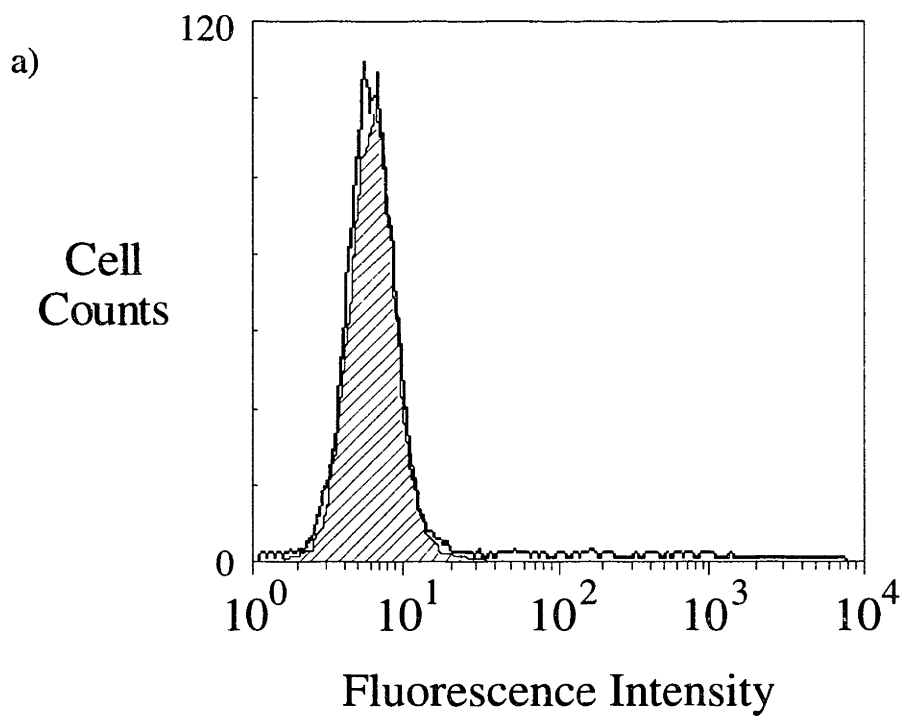
Figure 2.10 Demonstration of receptor-mediated delivery by FACS. a) complete histogram b) detailed view

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3. Cell Surface Binding and Conjugate Gene Delivery

3.1 Introduction

Although molecular conjugate vectors have significant promise, their efficiency must be substantially improved before they become effective *in vivo*. There is evidence that escape from the cell's endocytic network poses a obstacle to polyplex gene delivery, since the attachment of lysogenic peptides or adenoviral virions that disrupt endosomal membranes after internalization significantly enhances gene transfer efficiency (Cotten *et al.*, 1992; Curiel *et al.*, 1991; Plank *et al.*, 1994). However, a mechanistic study of the cell biology of gene transfer can reveal additional barriers to gene delivery, as well as identify means to improve conjugates further in order 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. The first is the net charge of the conjugate. The cell surface membrane is negatively charged due to its lipid, glycoprotein, and proteoglycan components (Mislick and Baldeschwieler, 1996). Therefore, since the net charge of molecular conjugates may determine the degree of binding due to specific receptor-ligand vs. nonspecific charge interactions, the overall ratio of polycation to nucleic acid in a conjugate preparation can significantly affect both delivery efficiency and specificity. Second, crosslinking a ligand to the conjugate 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.

Above we described the development of a receptor-mediated gene delivery system, and we now apply that system to identify rate-limiting steps to gene delivery. Here, we investigate whether polyplex binding to the cell surface is a rate-limiting step in gene

delivery. We show that the ligand crosslinker spacer length, the conjugate charge ratio, and the number of ligands present affect molecular conjugate binding, internalization, and overall gene delivery. Furthermore, we demonstrate that optimization of these properties can significantly enhance conjugate gene delivery efficiency and specificity.

3.2 Materials and Methods

3.2.1 Changes to Experimental System

Several modifications were made to the experimental system. First, NR6 mouse fibroblasts were used for all subsequent experiements instead of B82 fibroblasts, since gene delivery to the latter cells was not sufficiently reproducible to make them useful for quantitative studies. Wild type NR6 cells are devoid of EGFR, and cell lines that express wild type or a varety of receptor mutants have also been generated. Some experiments were also conducted in CV-1 cells. A second change was in the order of mixing components during conjugate formation; biotin-EGF is now added after the complexation of streptavidin-polylysine and DNA to ensure that all EGF is tethered near the conjugate surface. Finally, the plasmid pEGFP-C1 is used instead of phGFP-S65T in all subsequent experiments. The GFP expressed from the former plasmid, which has a F64L substitution in addition to the S65T, has been found to be a more sensitive reporter (Cormack et al., 1996).

3.2.2 Materials

Mouse EGF, penicillin-streptomycin, L-glutamine, trypsin-EDTA, nonessential amino acids, sodium pyruvate, G418, and MEMα medium were obtained from Life Technologies. Proteins were iodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) using Iodobeads and following the manufacturer's protocol (Pierce Chemicals). Unless otherwise mentioned, all other reagents were obtained from Sigma Chemicals.

3.2.3 Molecular Conjugate Preparation

Streptavidin crosslinking and purification were conducted essentially as described above. Briefly, polylysine of an average length of 180 residues was reacted for 1 h with an equimolar amount of SPDP in HBS (20 mM HEPES, pH 7.0, 150 mM NaCl). A 50% molar excess of polylysine was then reacted overnight at room temperature with streptavidin, containing approximately four maleimide groups per protein, in HBS with 1 mM TCEP. The crosslinked streptavidin-polylysine (SA-pK) was purified by sequential 2-iminobiotin affinity and HiTrap SP (Pharmacia Biotech) cation exchange chromatography. Streptavidin was quantified by absorbance at 280 nm and polylysine by the OPA Protein Assay (Pierce Chemicals) using pure polylysine as a standard. Three types of biotinylated EGF were synthesized by reacting mouse EGF in HBS with a tenfold molar excess of Sulfo-NHS-Biotin, Sulfo-NHS-LC-Biotin, or Sulfo-NHS-LC-LC-Biotin (Pierce Chemicals) followed by purification on a G-25 column and quantitation by absorbance at 280 nm using a Spectramax 250 spectrophotometer (Molecular Devices).

pEGFP-C1 plasmid DNA (Clontech, Palo Alto, CA), 4731 bp in length, was propagated in the DH5α E. coli strain and purified with a Plasmid Maxi Kit (Qiagen, Chatsworth, CA). 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 to the solution as it was vortexed, and the resulting complexes were extensively dialyzed against HBS using 1000 MWCO membrane (Spectrum). Biotinylated EGF was then added to the complexes, and the solution was incubated for 1 hour.

3.2.4 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 from 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 complexes were added to 0.165 nmoles of radiolabeled biotin-EGF in HBS and incubated for 1 hour at room temperature. The samples were then applied to 30,000 MWCO Ultrafree-MC Centrifugal Filter Units (Millipore Corporation) and centrifuged at 5,000 g, followed by two washes with 0.1% Triton X-100 in HBS. Bound radioactivity was then removed by washing with bleach, and ^{125}I-biotin-EGF was counted with a Packard 5000 series γ-counter (Packard Instruments).

The efficiency of biotinylation was tested for one reagent, Sulfo-NHS-LC-LC-Biotin. ^{125}I-Biotin-EGF in PBS was incubated with streptavidin immobilized to agarose (Sigma Chemicals) for one hour, both with and without 2 mM free biotin. The agarose was pelleted by centrifugation and washed twice with four volumes 0.1% Triton X-100 in PBS, and the amount of radioactivity bound to duplicate samples of the agarose was counted.

3.2.5 NR6 Cell Culture

Swiss 3T3-derived NR6 fibroblasts, which lack endogenous EGF receptors, have been transfected with a gene encoding the wild-type human EGFR and selected with G418, as described (Chen et al., 1994). Cells were maintained in 10 cm tissue culture dishes (Corning Glass Works) in MEMα medium supplemented with 7.5% FBS, penicillin (100 IU/ml) and streptomycin (2.5 μg/ml), 350 μg/ml G418, 2 mM L-glutamine, 100 mM sodium pyruvate, and 10 mM MEM nonessential amino acids in a humidified incubator (5% CO_2) at 37°C.

3.2.6 Molecular Conjugate Gene Delivery

For gene delivery experiments, 50,000 cells were seeded in 35-mm tissue culture dishes (Corning) and grown overnight. Cells were counted using a Coulter Multisizer II Counter (Coulter Electronics Ltd., England). Polyplexes containing 1 μg of DNA were then added to the cells in gene transfer medium, growth medium lacking FBS but instead containing 1% insulin-transferrin-selenium supplement (Life Technologies) plus 100 μM

chloroquine to yield a total volume of 1 ml. Excess mouse EGF was added to some samples at a concentration of 1 µg/ml. After a 6 h incubation, the conjugate solution was aspirated and replaced with regular growth medium. After 48 hours, the duplicate samples were trypsinized and analyzed for GFP expression using a FACScan flow cytometer (Becton Dickson, Franklin Lakes, NJ) with a 488-nm excitation laser and a 530/30-nm fluorescein emission filter.

3.2.7 Cell Surface Binding and Internalization Assays

Molecular conjugates were radioactively labeled with ^{125}I-SA-pK. SA-pK was iodinated to a specific activity of 64,700 cpm/ng of streptavidin and purified with SP Sepharose HP (Pharmacia Biotech), and 100,000 cpm were added per each µg of DNA at 10 µg/ml in 1 M NaCl, 20 mM HEPES while the solution was vortexed. 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 minutes at 37°C, centrifuged, and resuspended in gene transfer medium lacking sodium bicarbonate but buffered with 20 mM HEPES (pH 7.4). Radiolabeled conjugates containing 0.1 µg of DNA were added to approximately 50,000 cells in 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 pre-blocked with 10 mg/ml BSA. After loading of the cell suspensions and application of vacuum, the filters were washed five times with 0.2 ml of ice-cold WHIPS, 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 were added with 0.9 ml gene transfer medium to cells in a total volume of 1 ml, 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.

To determine the effects of multiple biotin-EGF occupancy of a single streptavidin protein on ligand binding, binding of conjugates to NR6 cells was conducted in the presence of biotinylated aprotonin. Sulfo-NHS-LC-LC-Biotin was added to 200 mg of aprotonin in HBS at a threefold molar ratio, and the reaction and purification were conducted as described above for EGF. 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.

3.2.8 Examination of Binding of Different Biotinylated EGF Molecules to EGFR

The biotinylated EGF yielding the highest binding of molecular conjugates to EGFR was determined by radioactive competition binding. Mouse EGF was iodinated to a specific activity of 160,000 cpm/ng and separated from free iodine with a G-25 column. NR6 cells were seeded at 100,000 cells per 35 mm tissue dish (Corning) and grown overnight. Conjugates were generated with each biotinylated EGF at a valency of 10. Duplicate cell samples were incubated in 1 ml of D/H/B (DME medium with no bicarbonate, 20 mM HEPES, pH 7.4, 1 mg/ml BSA) with 1 nM ^{125}I-EGF plus conjugates containing 1 µg of DNA. After incubation for 2 hours at 4°C, the cells were washed twice with 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). The cells were then solubilized with 1 N

NaOH, and the cell-associated radioactivity counted. Biotin-EGF with the longest spacer arm was used for all other experiments.

3.2.9 Measuring Cell Surface Receptor Levels

Cells were plated at 100,000 per 35 mm plate and grown overnight. Conjugates were then generated at valencies of 5, 15, and 50. Internalization experiments were conducted with these non-radiolabeled 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 essentially as described by Reddy et al. (Reddy et al., 1996). Ligand was stripped from the surface with two 2 min washes with 1 ml of ice-cold acidic salt strip, as above, 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 3 times with 2 ml of ice-cold WHIPS, solubilized with 1 N NaOH, and the radioactivity was counted.

3.3 Results

3.3.1 Determination of Maximum Biotin-EGF Capacity

The efficiency of the biotinylation reaction with one reagent, Sulfo-NHS-LC-LC-Biotin, was tested by quantifying the binding of radiolabeled biotin-EGF to streptavidin-agarose. $96.2 \pm 6.1\%$ of the radioactivity bound to the gel, while the presence of 2 mM biotin reduced the binding to $4.8 \pm 1.2\%$, where the error is the standard deviation 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 streptavidin-polylysine (SA-pK) at a ratio of 1.5 streptavidin molecules per polylysine chain. To determine the maximum amount of EGF that could bind to the streptavidin incorporated into the molecular conjugates, we generated conjugates at an electroneutral charge ratio and added increasing amounts to 0.165 nmoles of

radioactively labeled biotinylated EGF. The samples were centrifuged through a filter, and the biotin-EGF bound to conjugates was retained, while the free ligand was washed through. After counting the radioactivity retained on the filter due to the addition of 0, 10, 50, 100, 200, and 500 ng of DNA incorporated into conjugates, the moles biotin-EGF retained was plotted vs. the moles of DNA added (data not shown). The data fit the line:

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

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

3.3.2 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 conjugate preparations on gene delivery, binding, and internalization was determined. We conducted all experiments both in the presence and absence of 1 μg/ml mouse EGF to allow for the comparison of receptor-specific vs. nonspecific charge interactions between the conjugates and cells. Figure 1 shows that gene delivery is absent for low lysine/nucleotide ratios, where all errors are 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 only rises for lysine/nucleotide ratios above 1.3, and gene transfer efficiency is always much higher in the samples without excess ligand. Above a lysine/nucleotide ratio of 4, we observed cell toxicity. The same expression trends were observed in CV-1 cells (data not shown).

In order 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. We found that the association between radiolabeled SA-pK and DNA was stable to the addition of a large excess of polylysine and to the conditions used during incubations with cells (data not shown). We found a large degree of nonspecific binding of conjugates to tissue culture plastic (data not shown), so we conducted the binding in cell suspension in order to virtually eliminate background. After 3 h of agitation at 4°C, cells were separated from free conjugate using a filtration apparatus, and the dependence of cell binding on the charge ratio is shown in Figure 2. Assuming the ^{125}I-SA-pK was nearly equally distributed among all plasmid DNA molecules when they were mixed by vortexing, the approximate number of bound plasmids per cell is plotted vs. the lysine/nucleotide ratio. 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 significantly lower than the total binding only in the region around a lysine/nucleotide ratio of 1.

The same preparations of radiolabeled conjugates were also incubated with cells on 35 mm plates for six hours to measure endocytosis. To isolate only internalized counts, a pH 3, 2 M NaCl wash was required to remove radioactivity bound to the cell surface and tissue culture plastic. The low pH strips EGF from its receptor, while the salt dissociates the DNA and polylysine. The washes were found to remove >90% of bound radioactivity (data not shown). The cell surface membranes were not compromised during the washes, as the cells still excluded trypan blue (data not shown). Since chloroquine inhibits lysosomal degradation as well as EGF receptor recycling (Jiang and Schindler, 1990; King *et al.*, 1980; Stoscheck and Carpenter, 1984; Tseng *et al.*, 1992), the internal counts

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represent the sum of all internalization over the 6 h experiment. The internalization curves, shown in Figure 2, manifest the same trend as the binding curves, with internalization increasing with increasing lysine/nucleotide ratios to a maximum of over 400,000 per cell. In addition, as with binding, the curves with and without excess ligand differ significantly only near a ratio of 1.

3.3.3 Determination of the Optimal Biotinylated EGF

To determine whether crosslinking of EGF to the molecular conjugate could reduce EGF affinity for its receptor, presumably by sterically hindering their interaction, we generated and purified three different biotinylated EGF molecules using biotinylation reagents with progressively longer spacer arms. Their ability to mediate conjugate binding 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 radiolabeled EGF is reduced due to the presence of the molecular 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 radiolabeled EGF by 91%, while ones with the 13.5Å arm inhibited binding by only 10%.

Gene transfer was then conducted at a valency of 10, and the results, also shown in Table I, show that at this valency increased binding improves gene delivery efficiency. The conjugates generated with the smallest linker had an efficiency of 2.3%, while the longest linker yielded an 18% efficiency. Therefore, increasing the spacer arm length between EGF and biotin improves cell surface binding and gene delivery efficiency at low valency.

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3.3.4 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. Figure 3 shows the gene transfer efficiency as a function of ligand valency. The efficiency rises sharply at low valencies, goes through an optimum of 18% between a valency of 10 and 20, and then declines to below 2.5% between 20 and 50. The same trend in efficiency was observed in CV-1 cells (data not shown). We then investigated whether binding and internalization effects could account for this behavior. Binding and internalization experiments were conducted as above using complexes with varying valency, and results are shown in Figure 4. Both binding and internalization show the same maximum as gene delivery at a valency between 10 and 20, with binding peaking at 72,000 plasmids per cell and internalization at 137,000 plasmids per cell.

Reduced binding, internalization, and delivery at higher valencies could potentially be due to steric interference between multiple biotin-EGF proteins bound to the same streptavidin. To address this possibility, binding of conjugates at a valency of 15 was measured in the presence and absence of a large excess of biotinylated aprotonin, which is of molecular weight 6500 and therefore close in size to EGF. $49,100 \pm 1700$ complexes bound per cell in the absence and $46,900 \pm 4600$ in the presence of biotinylated aprotinin, where the errors are the standard deviations of two samples. Biotin-aprotonin likely occupies all the biotin-binding sites on the conjugate not occupied by biotin-EGF, but its presence makes no statistically significant difference in conjugate binding to NR6 cells. Therefore, steric hindrance between multiple EGF molecules bound to the same streptavidin at high valencies likely does not account for the drops in delivery, binding, and internalization observed.

Another possibility that could account for 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.

3.3.5 Measurement of Cell Surface EGF Receptor Levels

The reduction in the number of conjugates bound at high valencies, potentially due to receptor saturation, may lead to reduced 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 (Starbuck et al., 1990; Wiley, 1988). If endocytosis is saturated, then cells are 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 EGF receptors on the cell surface was measured by counting the amount of radioactivity bound to cells after incubation in a saturating amount of ^{125}I-EGF. A saturating level of free EGF should lead to the maximum potential rate of receptor internalization. In addition, the presence of chloroquine inhibits EGF receptor recycling after internalization (King et al., 1980; Stoscheck and Carpenter, 1984; Tseng et al., 1992); 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 downregulation rate. Figure 5 shows that with excess free EGF, the surface receptor number decreases approximately linearly from 380,000 per cell to 180,000 per cell by 6 h, or only $43 \pm 5.5\%$ of its original value. At a conjugate valency of 5, receptors are downregulated to only $71 \pm 3.4\%$, or 270,000 receptors per cell. However, at valencies of 15 and 50, EGF receptors are downregulated to $40 \pm 1.8\%$ and $42 \pm 8.4\%$ of their original levels. All errors are the standard deviations of two samples. The receptor downregulation due to conjugates at valencies of 15 and 50 is not statistically distinguishable from that observed for free EGF.

3.4 Discussion

We have investigated whether conjugate 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 have focused on three properties, or parameters, of the polyplexes that could potentially affect binding of the conjugate to the cell: the lysine/nucleotide ratio, the crosslinker used to attach the ligand, and the number of ligands added per plasmid. The effects of these parameters on cell surface binding, internalization, and gene delivery have been examined. For these studies, we employed an experimental system that uses EGF to direct delivery of pEGFP-C1 plasmid encoding GFP to mouse fibroblasts stably expressing the human EGF receptor. The DNA was complexed with polylysine crosslinked to streptavidin, followed by the addition of biotinylated EGF. Cells were incubated with the conjugates for 6 h, and GFP expression was analyzed 48 h later by FACS.

Adding increasing amounts of polycation added to a fixed amount of DNA changes the surface charge of polyplexes from negative, through electroneutral, to positive, as determined by measuring the zeta potential of the complexes (Legendre *et al.*, 1997; Wolfert, 1996). As expected, since the cell surface membrane is negatively-charged, positively-charged conjugates nonspecifically, ionically bind to the cell membrane, likely

due to interactions with proteoglycans (Mislick and Baldeschwieler, 1996). Therefore, if specific, receptor-mediated gene delivery is desired, it is important to know precisely how the 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 (Sosnowski *et al.*, 1996; Wadhwa *et al.*, 1995; Wagner *et al.*, 1990). However, the interpretation in most cases is complicated by the fact that as more crosslinked ligand-polylysine was added to the DNA, the number of ligands increased along with the charge ratio. In addition, the contributions of receptor-ligand vs. nonspecific ionic interactions to gene delivery were not separated.

We have 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. We found that binding and internalization increase as the lysine/nucleotide ratio is increased from 0.5 to 4, as shown in Figure 2. The internalization curves are steeper than the binding curves because, with inhibition of recycling and degradation due 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 one. Below a ratio of one, regardless of the presence of biotin-EGF, the negatively-charged conjugates are likely either ionically repelled by the cell membrane or not sufficiently condensed to be internalized . Conj ;ates generated at a ratio greater than one likely nonspecifically adsorb to the surface, also an effect independent of the presence 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. Even in this regime, however, the nonspecific contributions are surprisingly significant.

Gene transfer efficiency as a function of charge ratio, shown in Figure 1, follows similar trends to binding and internalization: delivery is always negligible at low charge ratios, and nonspecific delivery increases at high ratios. However, in contrast to binding

and internalization, at charge ratios greater than one gene delivery is always much higher if 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 that are 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 (French *et al.*, 1995). Alternatively, there are numerous reports of EGF receptor 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 (Cao *et al.*, 1995; Holt *et al.*, 1994; Holt *et al.*, 1995; Jiang and Schindler, 1990; Marti *et al.*, 1991). 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 the crosslinker used to tether the ligand to the conjugate can affect its affinity. Increasing the crosslinker arm length significantly increases the ability of biotinylated EGF tethered to polyplexes to compete with free, radiolabeled EGF for binding to the EGFR. It has been found that crosslinking of streptavidin molecules by biotin dimers requires a distance between the two biotins of at least 23 Å (Wilbur *et al.*, 1997), so that we would expect that 11.5 Å is the minimum length for effective binding of biotinylated EGF to the conjugates. All biotin reagents we used have arms greater than this distance, so the additional space likely provides 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

retroviral vectors displaying ligands fused to an envelope glycoprotein for retargeting delivery (Ager *et al.*, 1996; Valsesia-Wittmann *et al.*, 1996).

While the results from using different length crosslinkers 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, conjugates cannot bind efficiently, so internalization and gene delivery are low. Increasing valency improves binding, internalization, and delivery to an optimum at a valency near 15. Further increases in valency, however, lead to a rapid decrease in all three curves and an eightfold drop in efficiency. Binding likely decreases because, from valencies of 15 to 50, nearly all the 380,000 EGF receptors are likely 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, we find that the binding of 1 nM radiolabeled EGF decreases by as much as 91% in the presence of conjugates of valency 15 (Table 1). With the receptors saturated, increasing the number of ligands per conjugate only decreases the number of plasmids that can bind to each cell.

If a high number of EGF receptors is occupied, then EGF receptor-mediated endocytosis becomes saturated, meaning that the overall rate of receptor internalization reaches a maximum. Endocytic saturation is likely with conjugates of higher valency, since in NR6 cells it occurs at an EGFR occupancy of only 50,000 to 100,000 (J. Haugh, personal communcation). To test this possibility, we monitored the disappearance of EGFR from the cell surface. Conjugates at a valency of 5 downregulate the EGFR by only 29% over 6 h. In contrast, conjugates of valency 15 and 50 downregulate the receptor by over 65%, a result identical to the downregulation observed with 165 nM free EGF. Therefore, conjugates of valency 15 already drive the highest overall rate of receptor endocytosis, so that increasing valency further only decreases the number of plasmids carried inside the cell by the fixed number of internalized receptors. 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 (Douglas et al., 1996; Lee and Huang, 1996; Maruyama et al., 1995; Ohno et al., 1997; Remy et al., 1995; Watkins et al., 1997).

These experiments show that binding to the cell surface is a barrier to gene transfer, and that the conjugate can be engineered to increase delivery 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. Since nonspecific binding appears to improve transfer efficiency at high charge ratios, there may be a tradeoff between delivery efficiency and specificity, and the gene delivery application would likely determine the choice. In addition, there is a significant optimum in gene delivery as a function of conjugate affinity for the receptor. At low valency, affinity is insufficient for effective binding and internalization. However, increasing valency beyond an 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.

Crosslinker Name	Spacer Arm Length (Å)	Inhibition of EGF Binding	Gene Transfer Efficiency
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%

Table 3.1 Effects of Ligand Crosslinker Spacer Arm Length
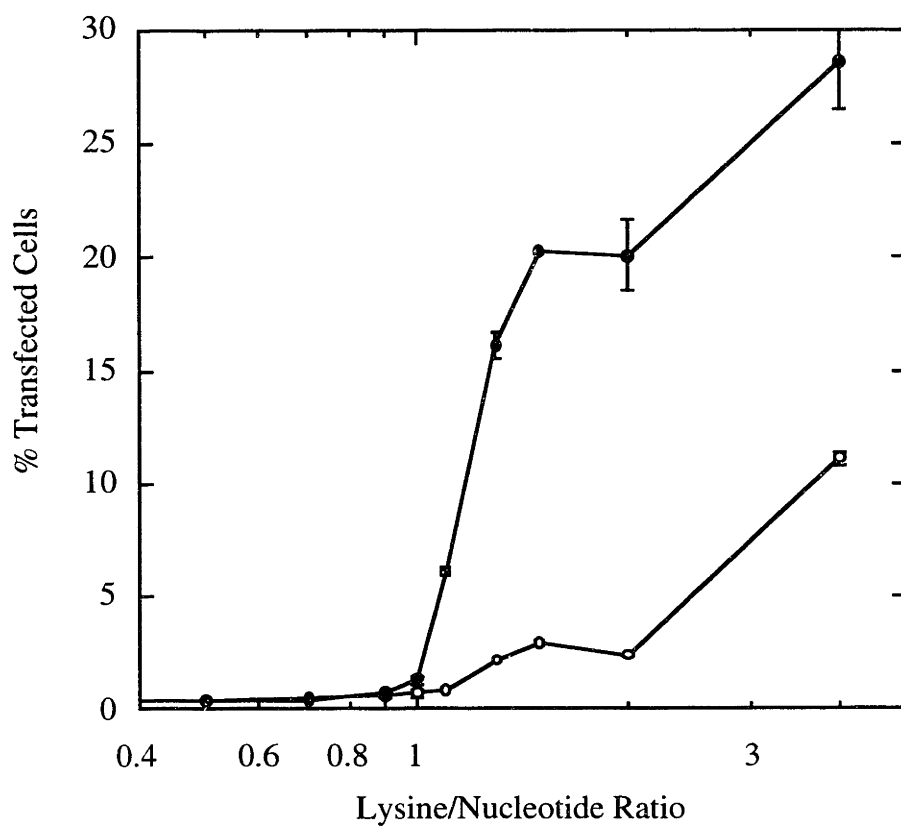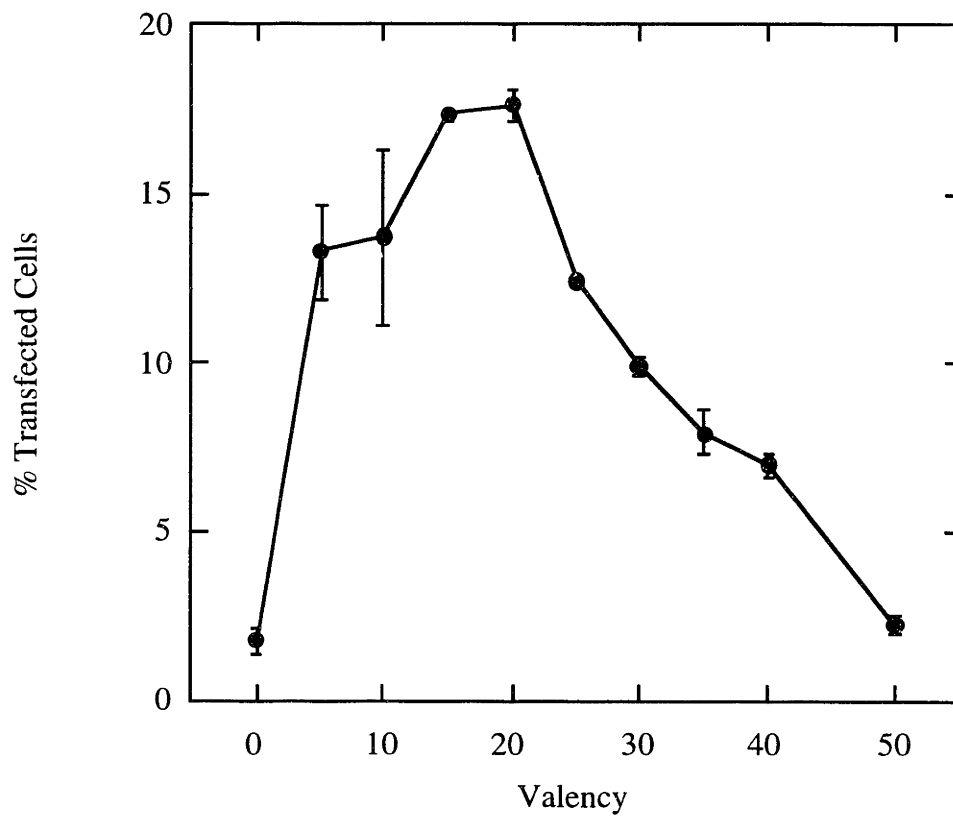
Figure 3.1 Gene transfer efficiency as a function of conjugate lysine/nucleotide ratio

Samples in the absence (●) and presence (○) of 1 μg/ml excess mouse EGF, to block conjugate interactions with the EGF receptor, were compared.
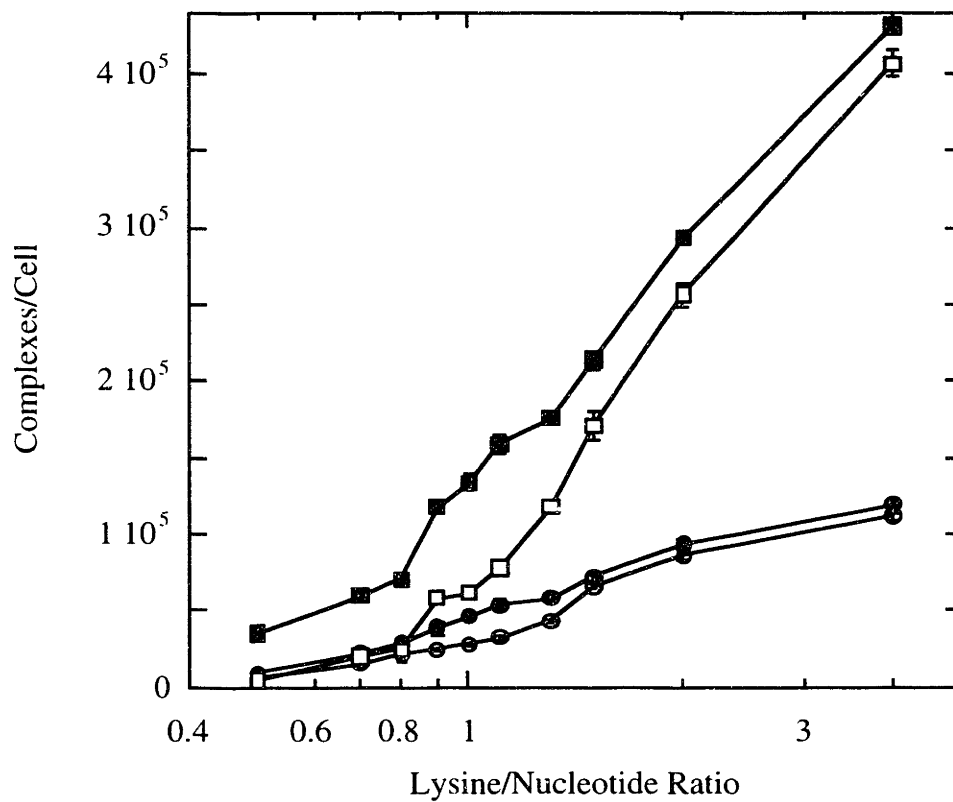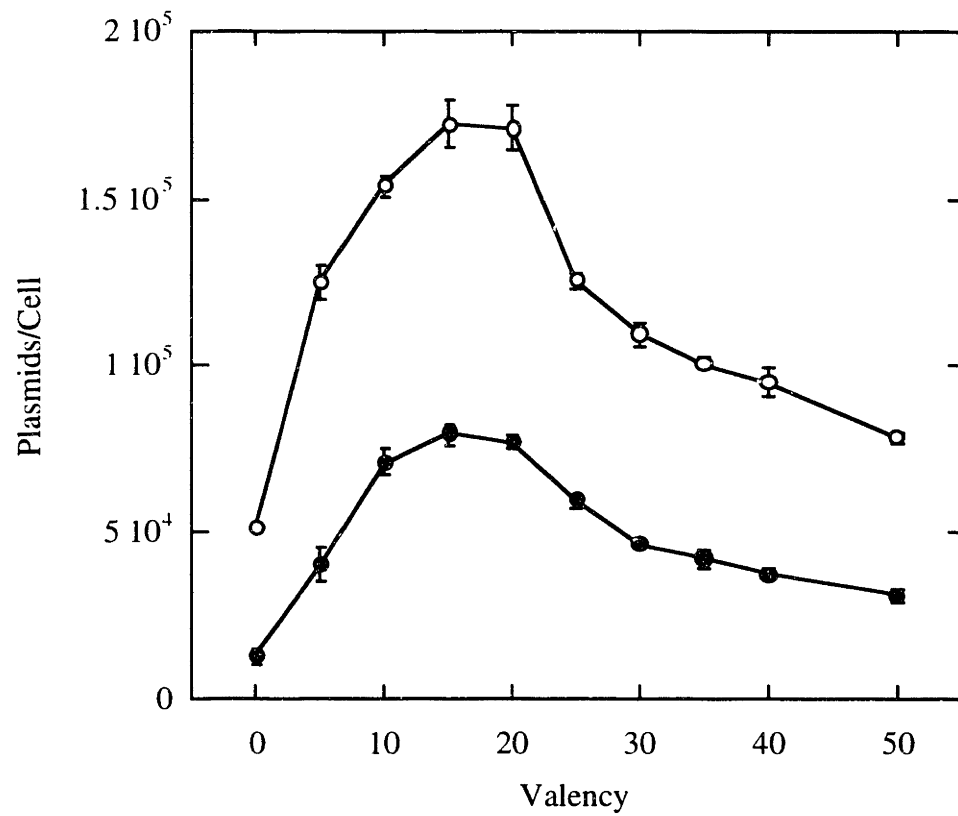
Figure 3.2 Binding and Internalization as a function of conjugate lysine/nucleotide ratio

Binding was compared in the absence (●) and presence (○) of 1 µg/ml excess mouse EGF, as was internalization, in the absence (■), presence (□) of excess EGF.

Figure 3.3 Gene transfer efficiency as a function of conjugate valency

Figure 3.4 Conjugate binding and internalization as a function of conjugate valency

The number of plasmids bound (●) and internalized (O) per conjugate is compared.
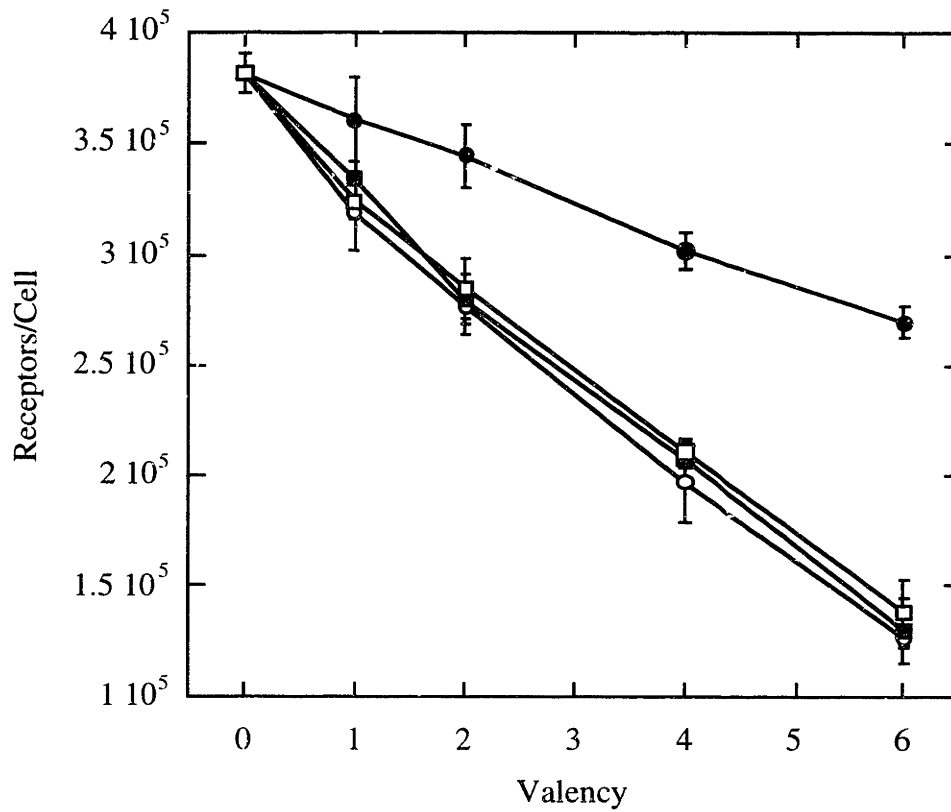
Figure 3.5 Dependence of receptor downregulation on conjugate valency

The number of remaining surface receptors is plotted vs. conjugate valency for conjugates of valency 5 (●), 15 (O), and 50 (■), as well as for 1 µg/ml EGF (□).

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4. Conjugate Unpackaging and Gene Delivery

4.1 Introduction

Improvements in efficiency can be achieved through a detailed examination of the mechanism of and identification of barriers to conjugate gene delivery. Previous work has demonstrated that binding to the cell surface (Schaffer and Lauffenburger, 1998), escape from the endosomal network (Cotten et al., 1992; Curiel et al., 1991; Plank et al., 1994), and translocation to the nucleus are rate-limiting steps for DNA transfer (Capecchi, 1980; Sebestyen et al., 1998). Engineering a vector to overcome these barriers can yield significant gains in delivery. In the work that follows, we investigate whether the final step of the gene delivery process, unpackaging of the polyplex, can limit the efficiency of gene delivery and expression. We report here that vector unpackaging appears to be a necessary step for gene expression, and that initial expression levels can be significantly increased by using a short polycation that releases the plasmid more rapidly.

4.2 Materials and Methods

4.2.1 Materials

Mouse EGF, penicillin-streptomycin, L-glutamine, trypsin-EDTA, nonessential amino acids, sodium pyruvate, G418, and MEMα medium were obtained from Life Technologies. Proteins were iodinated with $Na^{125}I$ (New England Nuclear) using Iodobeads and following the manufacturer's protocol (Pierce Chemicals). Unless otherwise mentioned, all other reagents were obtained from Sigma Chemicals.

4.2.2 Construction of pEG-1

pGEMEX-1 was obtained from Promega and pEGFP-C1 from Clontech, and the EGFP gene was cloned into pGEMEX-1 at its multiple cloning site. A 1619-nucleotide DNA fragment corresponding to a region of the pEGFP-C1 vector stretching from the

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CMV promoter to the SV40 polyadenylation site (1-1619 base pairs) was amplified by polymerase chain reaction using primers (Genemed Biotechnologies, South San Francisco, CA) containing an Sfi I site in the 5' and an Sph I site in the 3' overhang. The resulting PCR fragment was purified with a PCR Purification kit (Qiagen). pGEMEX-1 and the PCR fragment were then digested with Sph I and Sfi I (New England Biolabs, Beverly, MA), purified by agarose gel electrophoresis, and ligated using T4 DNA ligase (Life Technologies). DH5α *E. Coli* cells were transformed with the ligation reaction and plated on LB ampicillin plates, and positive colonies were identified by performing digests of miniprepped DNA. All plasmids were propagated in the DH5α cells and purified with a Plasmid Maxi Kit (Qiagen). Reactions and DNA manipulations were conducted as described in Ausubel *et al* (Ausubel et al., 1995).

4.2.3 NR6 Cell Culture

Swiss 3T3-derived NR6 fibroblasts, which lack endogenous EGF receptors, have been transfected with a gene encoding the wild-type human EGFR and selected with G418, as described (Chen et al., 1994). Cells were maintained in 10 cm tissue culture dishes (Corning Glass Works) in MEMα medium supplemented with 7.5% dialyzed FBS, penicillin (100 IU/ml) and streptomycin (2.5 μg/ml), 350 μg/ml G418, 2 mM L-glutamine, 100 mM sodium pyruvate, and 10 mM MEM nonessential amino acids in a humidified incubator (5% CO_2) at 37°C.

4.2.4 Fluorescence Microscopy of Polyplex Gene Delivery

Streptavidin-polylysine crosslinking and purification were conducted essentially as previously described (Schaffer et al., 1997). Briefly, polylysine of an average length of 35 residues was reacted with an equimolar ratio of SPDP in HBS (20 mM HEPES, pH 7.0, 150 mM NaCl) for 1 h. A 50% molar excess of the polylysine was then reacted with streptavidin-maleimide, containing approximately 4 maleimide groups per protein, in HBS with 1 mM TCEP overnight at room temperature. The crosslinked streptavidin-polylysine

(SA-pK) was purified by sequential 2-iminobiotin affinity and HiTrap SP (Pharmacia Biotech) cation exchange chromatography. Streptavidin was quantified by absorbance at 280 nm and polylysine by the OPA Protein Assay (Pierce Chemicals) using pure polylysine as a standard. Biotinylated EGF was synthesized by reacting mouse EGF in HBS with a tenfold molar excess of Sulfo-NHS-LC-LC-Biotin (Pierce Chemicals), followed by purification on a G-25 column and quantitation by absorbance at 280 nm using a Spectramax 250 spectrophotometer (Molecular Devices).

Polylysines of average length 19, 36, and 180 residues (pK19, pK36, and pK180) were fluorescently labeled with Alexa 488 following the manufacturer's instructions (Molecular Probes, Eugene, OR). After 0.5 mg of each polymer was labeled, it was separated from free reagent using Sepharose SP cation exchange gel (Pharmacia Biotech, Piscataway, NJ). The degree of labeling was determined by measuring absorbance at 494 nm to determine dye content. Polylysine was quantified using ninhydrin and measuring absorbance at 570 nm, since the dye appeared to interfere with the OPA assay. pGEMEX-1 was biotinylated using psoralen-biotin reagent following the manufacturer's instructions (Ambion, Austin, TX), and the DNA was separated from excess biotinylation reagent by dialysis vs. HBS using 10,000 MWCO membrane (Spectrum). One half mg of streptavidin was labeled with Alexa 546 (Molecular Probes) and separated from free reagent by extensive dialysis vs. HBS using 10,000 MWCO membrane. Biotinylated DNA was then incubated with a tenfold excess of fluorescently-labeled streptavidin for 1 h, and free biotin-binding sites were then blocked by addition of excess biotin. DNA was then separated from unlinked streptavidin and excess biotin by extensive dialysis vs. HBS using 300,000 MWCO membrane (Spectrum).

To form polyplexes, fluorescently-labeled plasmid was added to a 20 mM HEPES (pH 7.0), 1 M NaCl solution at a concentration of 10 µg/ml. Streptavidin-polylysine and fluorescently labeled polylysine were mixed at a lysine molar ratio of 1:4. This mixture was then added to the DNA solution while the solution was vortexed, and the resulting

complexes were extensively dialyzed against HBS using 1000 MWCO membrane (Spectrum). Loss of conjugate during dialysis was negligible. Biotinylated EGF was then added to the complexes at a valency, or ligand to plasmid ratio, of 15, and the solution was incubated for 1 h.

NR6 cells were plated at 5,000 cells/cm^2 on glass coverslips and grown overnight. Complexes were then added to cells in gene transfer medium, growth medium lacking FBS but instead containing 1% insulin-transferrin-selenium supplement (Life Technologies) plus 100 μM chloroquine. After a 6 h incubation, the conjugate solution was aspirated and replaced with regular growth medium lacking phenol red. After 48 h, samples were fixed with 4% paraformaldehyde, stained with 0.25 μg/ml DAPI (Molecular Probes) in PBS with 0.1% Triton, and mounted in Prolong Antifade reagent (Molecular Probes). Samples were visualized using a Nikon Eclipse E-800 fluorescence microscope with a 100x objective and a mercury arc lamp light source. The excitation light was passed through the appropriate filter on a filter wheel, and emission light was filtered with a triple filter emission cube. A z-series of typically nine images with a spatial separation of 0.25 μm was collected using a Photometrics AT200 cooled CCD camera system and Scanalytics Cellscan software run on a Dell PC. The images were then deconvoluted using Scanalytics Exhaustive Photon Reassignment software to generate planar images. Samples lacking Alexa 488, Alexa 546, or both labels were also visualized to analyze background levels of fluorescence.

4.2.5 *In Vitro* Measurements of Conjugate Unpackaging

The dissociation of polylysine of different lengths from plasmid DNA due to cation exchange was analyzed *in vitro*. Double-stranded DNA immobilized to cellulose was swelled and then washed extensively with HBS. Conjugates lacking fluorescent label were generated as described above, and in each sample approximately 50 ng of DNA incorporated into conjugates was mixed with 10 μl of the DNA-cellulose in a total volume of 60 μl of HBS. Samples were agitated at 37°C for various times and centrifuged, and the

supernatant was collected. Ten µl of each sample were then run on a 1% agarose gel using TAE as the buffer. Gels were stained with SYBR Gold (Molecular Probes) and visualized with a Fluor-S fluorescence imager (Bio-Rad Laboratories). Ten µl of the each of the 48 h samples were also run on the gel after complete dissociation of polylysine from the plasmid by the addition of 2 M NaCl, and plasmid loss during the experiment was found to be negligible.

4.2.6 *In Vitro* Transcription

Polyplexes were generated essentially as described above using either pEG-1 or pGEMEX-1 and either streptavidin-polylysine or uncrosslinked polycations. Biotinylated EGF was also added to some complexes that contained streptavidin. Conjugates were extensively dialyzed into 10 mM Tris buffer (pH 8.0), and there was negligible loss during dialysis. Conjugates containing approximately 0.12 µg of DNA were then added to a 30 µl transcription reaction assembled using a T7 RNA Polymerase *In vitro* Transcription Kit (Ambion, Austin, TX). For visualization, RNA was electrophoresed on a formaldehyde agarose gel (Ausubel et al., 1995), stained with Radiant Red (Bio-Rad), and visualized with a Fluor-S fluorescence imager (Bio-Rad). RNA molecular weight markers were obtained from Ambion. For accurate quantitation of RNA, reactions containing 0.2 µCi α32P-UTP (New England Nuclear) were run for 4 h at 37°C, and RNA was separated from free mononucleotides using 30,000 MWCO Ultrafree-MC Centrifugal Filter Units (Millipore Corporation, Bedford, MA). After two washes with 0.5 mL HBS, the radioactivity retained on the filter was counted using a Packard Tri-Carb 2500 TR liquid scintillation counter (Packard Instruments). *In vitro* transcriptions were also performed using polyethyleneimine with an average of 46 and 1200 monomers (Aldrich Chemicals).

4.2.7 Molecular Conjugate Gene Delivery

For gene delivery experiments, 50,000 cells were seeded in 35-mm tissue culture dishes (Corning) and grown overnight. Polyplexes lacking any fluorescent label and

containing either pEG-1 or pEGFP-C1 were generated as described above. Conjugates

containing 0.38 pmol of DNA were then added to the cells in gene transfer medium. After

a 6 h incubation, the conjugate solution was aspirated and replaced with regular growth

medium. After 48 h, the duplicate samples were trypsinized 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. Delivery was also conducted in the

presence of 1 µg/ml EGF to block binding of conjugates to the EGF receptor.

4.2.8 Quantitation of Internalized Conjugate

To quantify the internalization of conjugates composed of different sized

polylysines, experiments were conducted with radiolabeled conjugates as described

elsewhere (Schaffer and Lauffenburger, 1998). Cells were seeded at 100,000 per 35 mm

plate and grown overnight. Briefly, 100,000 cpm of ^{125}I-labeled SA-pK, iodinated to a

specific activity of 65,000 cpm/ng of streptavidin and purified with SP Sepharose HP

(Pharmacia Biotech), were added for each µg of 10 µg/ml pEG-1 in 1 M NaCl, 20 mM

HEPES while the solution was vortexed. Conjugates were then generated as described

above. and 1 µg of DNA in gene transfer medium with 100 µM chloroquine was added to

cells for 6 h. Cells were then washed twice 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. Cells were then solubilized with 1 N NaOH,

and the internalized radioactivity counted with a Packard 5000 series γ-counter (Packard

Instruments).

4.3 Results

4.3.1 Construction of pEG-1

Because the affinity of polylysine for DNA depends upon its sequence (Shapiro et

al., 1969), we generated one plasmid that could be used for nearly all experiments in this

study. pEGFP-C1 contains the double site-directed mutant of GFP with improved sensitivity over wild-type protein, while pGEMEX-1 contains a T7 promoter and terminator with a MCS just downstream of the promoter. The CMV promoter, GFP sequence, and SV40 polyadenylation site were inserted into pGEMEX-1 to generate the plasmid pEG-1. As shown in Figure 1a, digestion of pEG-1 with Sph I yielded a fragment of approximately 5500 bp (Lane 2), as expected from a theoretical length of 5565 bp, and digestion with Sph I and Sfi I generated two fragments of approximately 1600 and 4000 bp (Lane 3), corresponding to the GFP insert and pGEMEX-1 backbone. Digestion of pGEMEX-1 with Sph I yields a 3995 bp fragment (Lane 4). pEG-1 retains the ability direct expression of GFP in NR6 cells after transfection using LipofectAMINE (Life Technologies); however it is less sensitive than pEGFP-C1, since it is expressed at lower levels as assayed by FACS (data not shown).

In addition to the CMV promoter and SV40 polyadenylation site, pEG-1 contains a T7 RNA polymerase promoter and transcriptional terminator; therefore, both T7 and RNA polymerase II transcription can proceed through the GFP coding region of a circular plasmid. Addition of pEG-1 to an *in vitro* transcription reaction yields an approximately 2700-nucleotide RNA transcript initiated at the promoter, encompassing the GFP and T7 gene 10, and ending at the T7 transcriptional terminator. The RNAs generated from pEG-1 and pGEMEX-1 by T7 polymerase, electrophoresed on a formaldehyde agarose gel, are compared in Figure 1b. The majority of the RNA transcribed from pEG-1 was 2700 nucleotides long; however, a small fraction of elongations proceeded through the terminator to yield an approximately 8250-nucleotide transcript after termination on the second pass (Lane 3). In addition, the difference in length between transcripts from pEG-1 and pGEMEX-1 is approximately 1600 nucleotides, corresponding to the length of the GFP insert (Lane 4).

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4.3.2 Fluorescent Tracing of Conjugate Gene Delivery

By fluorescently labeling both the polylysine and the DNA, in addition to staining the nucleus, we were able to study in detail the localization of the conjugates within the cell. Polylysines were labeled with Alexa 488, resulting in the incorporation of one dye for every 40-50 lysine residues. After streptavidin was labeled at an Alexa 546 dye/protein ratio of 5.4, biotinylated pGEMEX-1 plasmid was mixed with an excess of the streptavidin. A typical preparation of purified DNA contained 8.1 streptavidin molecules per plasmid. We find that labeling with Alexa 546 dramatically enhances sensitivity over other DNA labels such as ethidium monoazide. Furthermore, the use of other sensitive DNA intercalating dyes such as YOYO-1 (Molecular Probes) is not suitable for this application, since they are not covalently linked and therefore dissociate from the plasmid under the conditions of the experiment. Finally, pGEMEX-1 was used for these studies since expression of GFP from pEG-1 would interfere with fluorescence tracing of the conjugates.

The DNA was complexed with fluorescently labeled polylysine and streptavidin-polylysine at a streptavidin/plasmid ratio of 29. After incubation with cells, samples were analyzed by fluorescence microscopy, and the images were deconvoluted to generate planar sections of cells. Cell samples were also generated from conjugates lacking either or both the polylysine and DNA labels to measure background fluorescence, and this background noise was found to be insignificant in all cases.

Figures 2-4 show typical planar images obtained for cells incubated with conjugates containing pK19, pK36, and pK180, where colocalization of polymer and plasmid in the overlaid images is shown as yellow regions. In all cases, both the polylysine and DNA colocalize to a large extent in cytoplasmic vesicles of the endocytic network, though the signal from pK19 always appears weaker. Even 48 h after delivery, however, the DNA signal in the perinuclear vesicles appears intense. In addition, in some cells we observed plasmid DNA within the nucleus, shown with an arrow, which is visualized by staining

with DAPI. In all samples observed with nuclear plasmid, pK180 colocalizes with plasmid, while pK19 and pK36 are never detected in the vicinity of the nuclear plasmid DNA.

4.3.3 *In Vitro* Conjugate Unpackaging

Experiments were conducted *in vitro* to determine whether polyplexes can unpackage due to cation exchange of the polylysine to a large excess of DNA, and whether there are measurable differences in the relative rates of dissociation with polylysines of different lengths. Conjugates were added to an approximately 800-fold higher amount of double stranded DNA immobilized to cellulose. Samples were agitated for various lengths of time at 37°C, separated from the excess DNA by centrifugation, and analyzed on an agarose gel. As shown in Figure 3, pK19 completely dissociates from the plasmid by 4 h, and pK36 by 8 h. In contrast, even after 48 h pK180 is only approximately 60% dissociated from the plasmid DNA.

4.3.4 *In Vitro* Transcription

Conjugates containing different lysine/nucleotide ratios were generated and dialyzed into 10 mM Tris, pH 8.0. Volume gain during dialysis was consistently less than 10%, and conjugate loss was negligible. Approximately 120 ng of DNA incorporated into conjugates was added to 30 μl T7 RNA polymerase *in vitro* transcription reactions, in order to model the relative rate of transcription from different conjugates in cells.

Figure 4a shows that both pK180 and pK36 significantly inhibit gene transcription. From a lysine/nucleotide ratio of 0 to 0.8, transcription decreases slightly. However, it decreases rapidly between a ratio of 0.8 and 1, and for all values above a ratio of 1 transcription levels are not above background. In contrast, for charge ratios up to 5, pK19 only reduces transcription to 60%, and further addition of the polylysine to a ratio of 15 decreases transcription only slightly further. The presence of streptavidin-polylysine or biotin-EGF does not alter the results (data not shown). The polycations likely inhibit

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transcript initiation and not elongation, since full length transcripts are generated in the presence of polylysine (data not shown). As shown in Figure 4b, both pK180 and pK19 stably bind DNA, as determined by their ability to retard the electrophoretic migration of DNA at similar lysine/nucleotide ratios. Finally, transcriptional inhibition does not appear to be due to DNA precipitation or formation of large aggregates, since centrifugation for 5 min at 1000g did not pellet the complexes.

We also performed gene transcription with templates condensed with polyethyleneimine, and Figure 5a shows that this polycation also inhibits transcription. In addition, as with polylysine, the smaller polymer does not inhibit to as large an extent as the large one. Figure 5b shows that both of these polymers form complexes with DNA that are stable under the conditions of electrophoresis.

4.3.5 Molecular Conjugate Gene Delivery

Conjugates lacking any fluorescent label were incubated with cells for 6 h, and gene delivery was quantified 48 h later by FACS analysis of GFP expression. Both pEG-1 and pEGFP-C1 were delivered. As shown in Table 1, conjugates generated with pK36 have the highest efficiency, up to 38%, with pK19 slightly lower and pK180 2.5-fold lower. Expression from pEGFP-C1 is detected in a higher fraction of cells, likely related to our finding above that it is more sensitive than pEG-1; however, the relative delivery efficiencies with the different polymers are the same with either plasmid. In addition, the delivery is receptor-mediated, since the presence of saturating EGF significantly blocks expression. This difference in efficiency could arise from differences in the cellular uptake of conjugates generated with the different polylysines. To test this possibility, we measured conjugate internalization and found that a similar amount of conjugate is internalized regardless of the polylysine length (Table 1). Using fluorescence microscopy, with all conjugates we visualized nuclear localization of plasmid in fewer than 10% of the cells, a low number compared to the observed gene delivery efficiencies. Thus, the

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microscopy may not have been sufficiently sensitive to detect all nuclear localization events. Alternatively, the fluorescent label may partially interfere with conjugate delivery.

4.4 Discussion

For *in vivo* polyplex gene delivery, the polycation protects the DNA from nuclease digestion and condenses it to a size that is more easily internalized by target cells. However, once inside the nucleus, in order to be recognized as nucleic acid by RNA transcription complexes, the DNA may first need to dissociate from the polycation. By using three polylysines with different affinities for DNA, we have investigated whether this dissociation step poses a barrier to transgene expression.

In this first use of fluorescence microscopy to trace both the polycation and plasmid DNA during polyplex gene delivery, we find the polylysine and DNA remain largely colocalized in cytoplasmic vesicles. However, for conjugates that succeed in escaping the endosome and localizing to the nucleus, the fate of the polycation depends upon its degree of polymerization. We find that pK180 remains colocalized with nuclear plasmid. In contrast, we find no pK19 or pK36 in the vicinity of nuclear plasmid, and it is likely that it is completely dissociated from the plasmid, and its signal therefore too diffuse to be observed. It is not known at what stage after internalization dissociation of the smaller polymer occurs, but the contrasting behavior may be due to differences in the affinities or the rates of dissociation of the polycations from the plasmid within the nucleus. To test this possibility, we incubated conjugates in the presence of a large excess of double-stranded DNA and found that pK19 and pK36 dissociate from the plasmid much more rapidly than pK180. This result indicates that a slower rate of cation exchange of the polylysine to the surrounding chromatin could account for the continued association of pK180 with plasmid DNA observed in the microscopy results.

We next investigated the potential consequences of association between the polymer and DNA in the nucleus. We measured transcription from the T7 promoter of plasmid

complexed with polylysine and found that the polycation significantly inhibits RNA synthesis. Transcription from DNA condensed at an electroneutral charge ratio with pK36, pK180, or polyethyleneimine with 1200 monomers is not above background. In contrast, inhibition by pK19 or polyethyleneimine with 46 monomers is significantly less. This may be due in part to the presence of 2 mM spermidine in the *in vitro* transcription buffer, since this compound's three protonated amines may dissociate the smaller polymers from the DNA. Both spermidine and spermine are present intracellularly at concentrations in the millimolar range (Cohen, 1971; Tabor and Tabor, 1984); therefore naturally occurring polyamines may play a role in dissociating polyplexes in addition to possible cation exchange of polylysine to chromatin. Inhibition of both T7 and RNA polymerase II transcription has also been observed with DNA condensed into polyplexes by cationic dendrimers (Bielinska et al., 1997). In addition, complexation with polylysine has also been found to inhibit DNA replication (Saffhill and Itzhaki, 1975).

Since the larger polycation both remains associated with the plasmid in the nucleus at 48 h and inhibits *in vitro* transcription, it would be predicted to lead to lower levels of gene expression. We find that 48 h after gene delivery GFP is expressed in 33% and 38% of cells using conjugates with pK19 and pK36, respectively. With polyplexes generated with pK180, however, GFP expression is detected in only 15% of the cells. Since dissociation of pK180 from DNA appears to be slow, gene expression from the plasmid may be inhibited by association with pK180, leading to detectable expression in fewer cells. Higher expression at short times after delivery with smaller polylysine has also been observed in hepatocytes.[*] Wu *et al.* have found that conjugate DNA can persist in cytoplasmic vesicles, and our findings suggest that conjugates can exist unpackaged in the nucleus. It has been proposed that persistent DNA can be slowly released and thereby contribute to prolonged transgene expression, but any potential effects of the polycation size on the duration of expression remain to be determined.

[*] A. Ziady, T. Ferkol, P. Davis, Personal Communication.

There is evidence that conjugate dissociation may play a role in endosomal escape of DNA. For example, polycations that function as proton sponges likely dissociate from DNA in the acidic endosomal network, and this dissociation has been proposed to be the mechanism for the ability of these polycations to enhance endosomal release (Boussif et al., 1995; Haensler and Szoka, 1993). In addition, the enhancement of conjugate gene delivery by chloroquine was thought to be due to its ability to inhibit lysosomal transport and degradation. However, chloroquine was found to dissociate polylysine from DNA, and this dissociation may stimulate endosomal escape in the same way as with proton sponges (Erbacher et al., 1996). Finally, it has been observed that neutralization of some of the positive charges of polylysine decreases its affinity for DNA and increases gene expression (Erbacher et al., 1997).

Partial or total dissociation may therefore occur within the endosome and/or later by cation exchange in the nucleus, but our results suggest that dissociation at some stage is required for efficient expression. There have been analogous findings in viruses: viral uncoating is a necessary step for replication. For example, the parvovirus MVMp replicates in fibroblasts but not lymphocytes, while the closely related virus MVMi has the opposite tropism. However, both capsids are transported to lymphocyte nuclei, and the lack of MVMp infection in these cells appears to be due to the inability of its capsid to uncoat in these cells (Previsani et al., 1997). Additionally, a cowpea chlorotic mottle virus with a single arginine to cysteine substitution in its coat protein is noninfective because disulfide bonding in the capsid blocks viral uncoating (Fox et al., 1997). A similar phenomenon is seen with poliovirus and reoviruses, since binding of antibodies to their capsids blocks uncoating and thereby prevents infection (Virgin et al., 1994; Wetz, 1993). In each case, the virus is highly infective under normal conditions; therefore, efficient unpackaging may be one more lesson that synthetic vectors should learn from viruses.

Polylysine Length	pEG-1 Gene Delivery	pEGFP-C1 Gene Delivery	Delivery with Excess EGF	Internalized Plasmids/Cell
19	16.9 ± 1.1%	32.5 ± 2.5	1.9 ± 0.05%	110700 ± 6100
36	22.0 ± 3.3%	37.7 ± 1.8%	1.7 ± 0.5%	116200 ± 2800
180	7.5 ± 0.8%	15.3 ± 0.6%	1.8 ± 0.3%	124900 ± 5700

Table 4.1 Gene delivery efficiency and internalization of conjugates generated with different polylysines
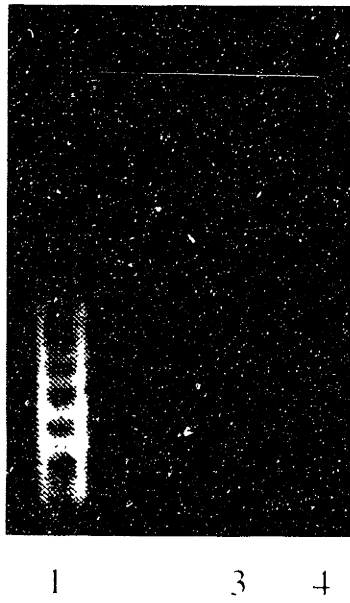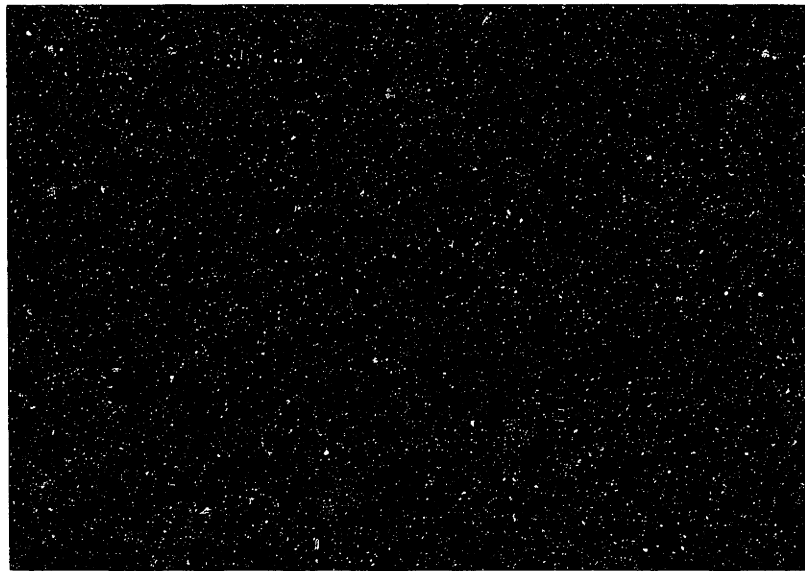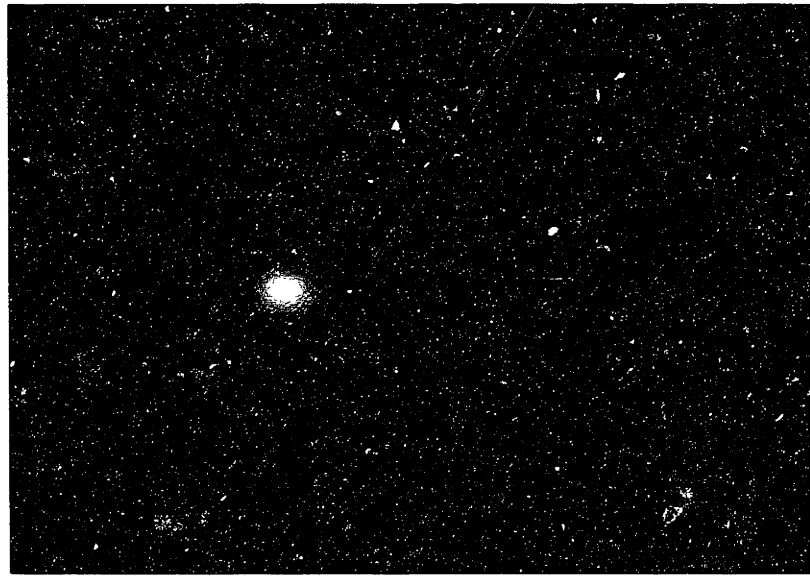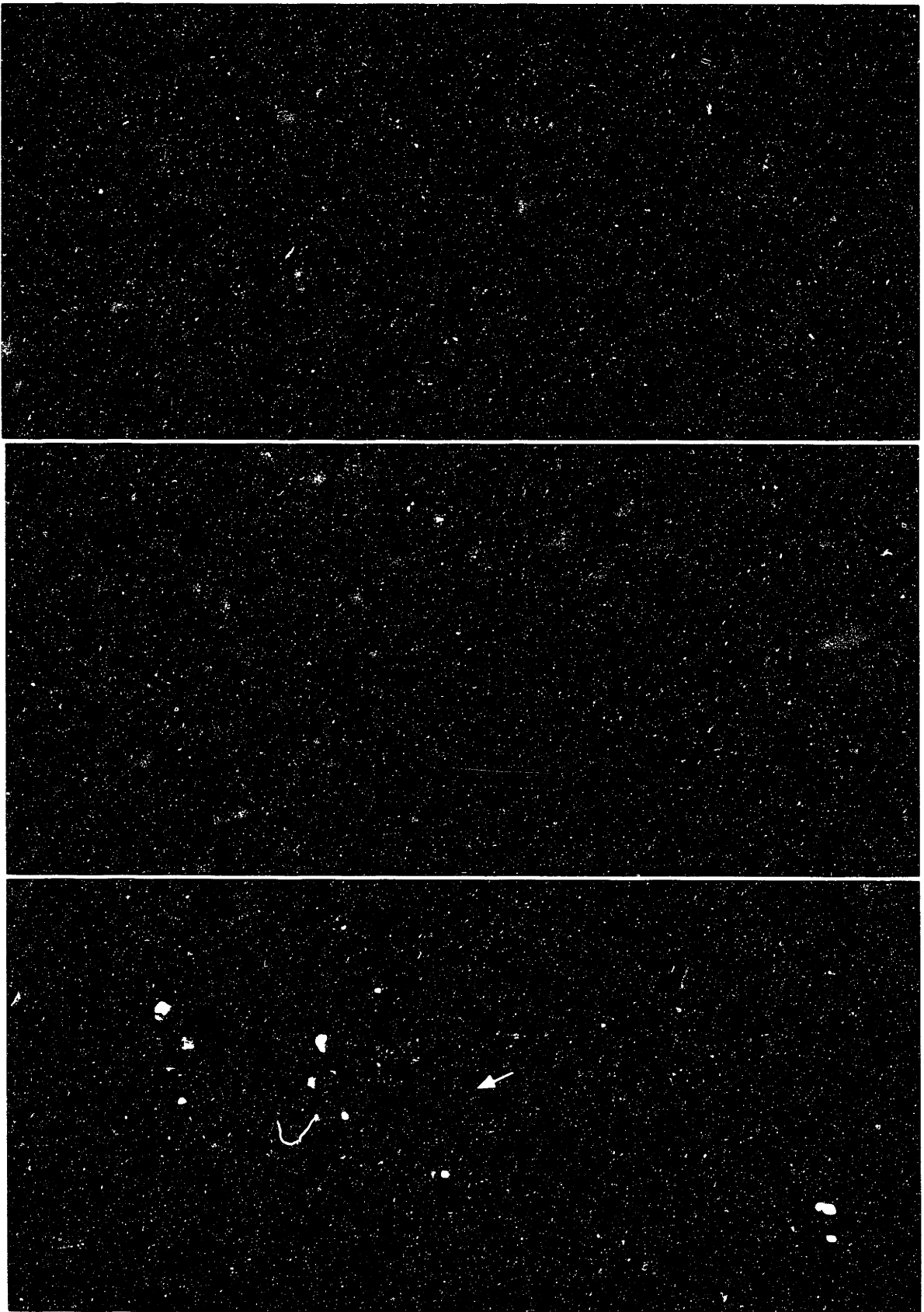
a)

1   2   3   4

b)

1   3   4
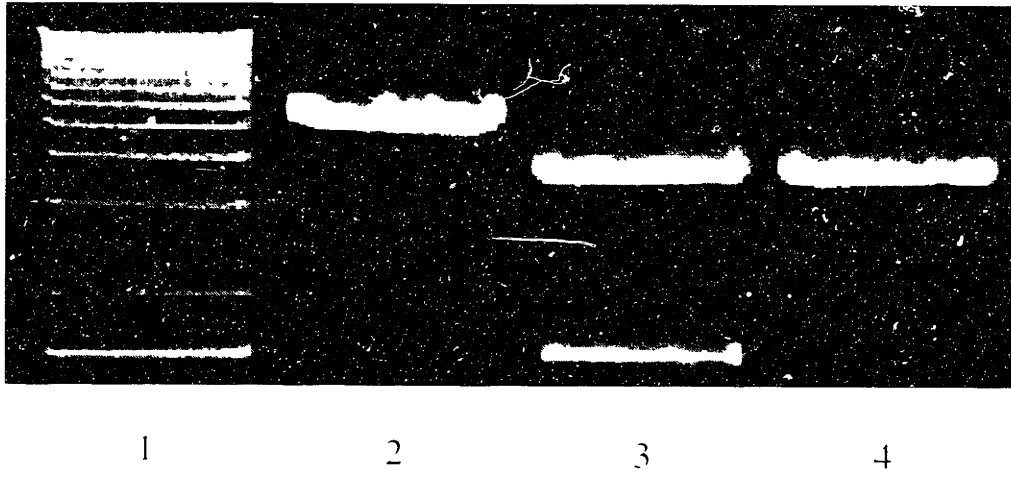
Figure 4.1 pEG-1 plasmid a) restriction digest b) *in vitro* transcription

Figure 4.2 pK19 fluorescence microscopy a) polylysine b) plasmid c) colocalization

Figure 4.3 pK36 fluorescence microscopy a) polylysine b) plasmid c) colocalization
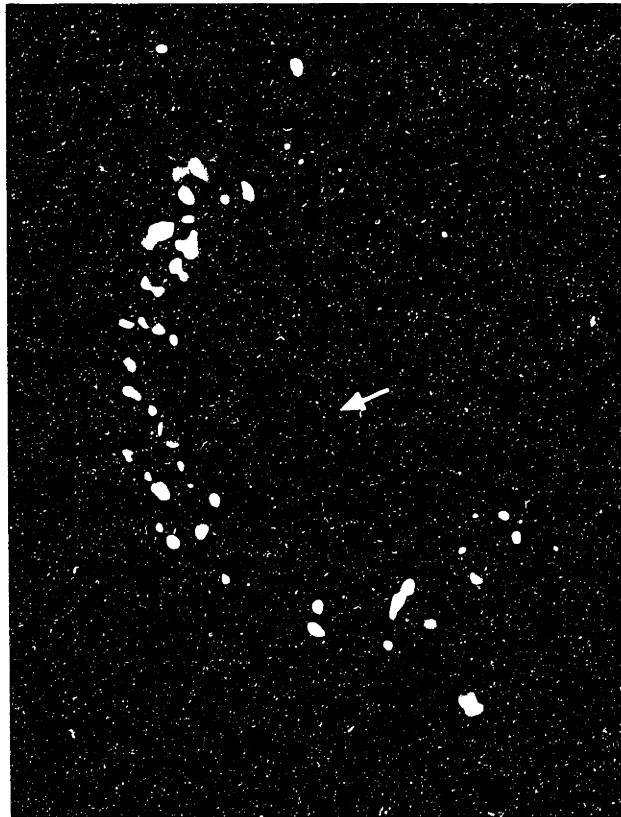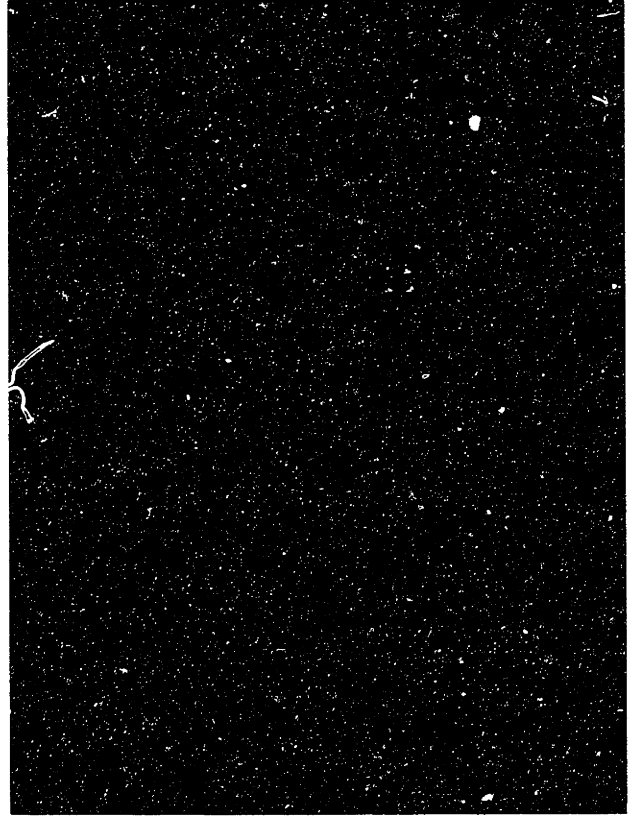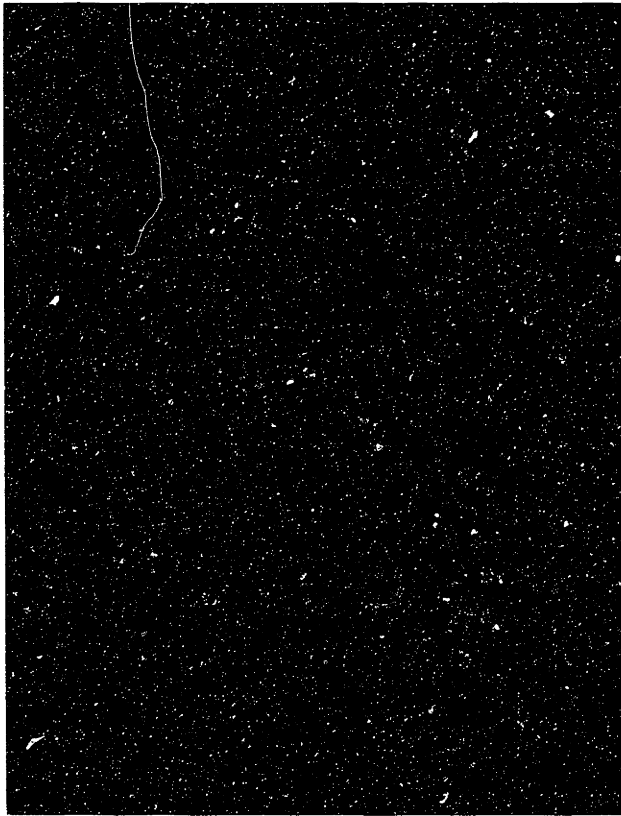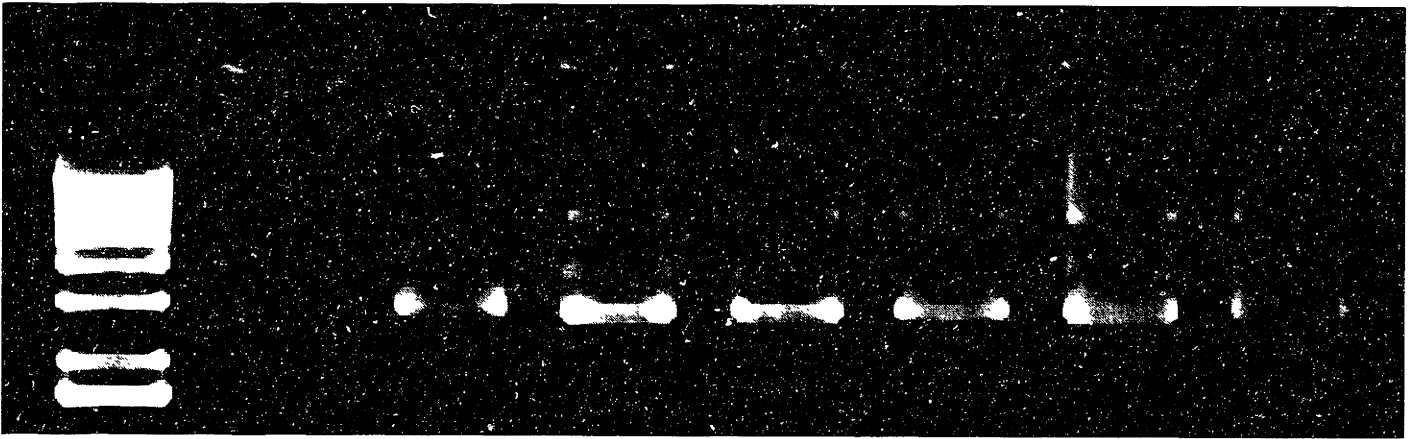
Figure 4.4 pK180 fluorescence microscopy a) polylysine b) plasmid c) colocalization

a)
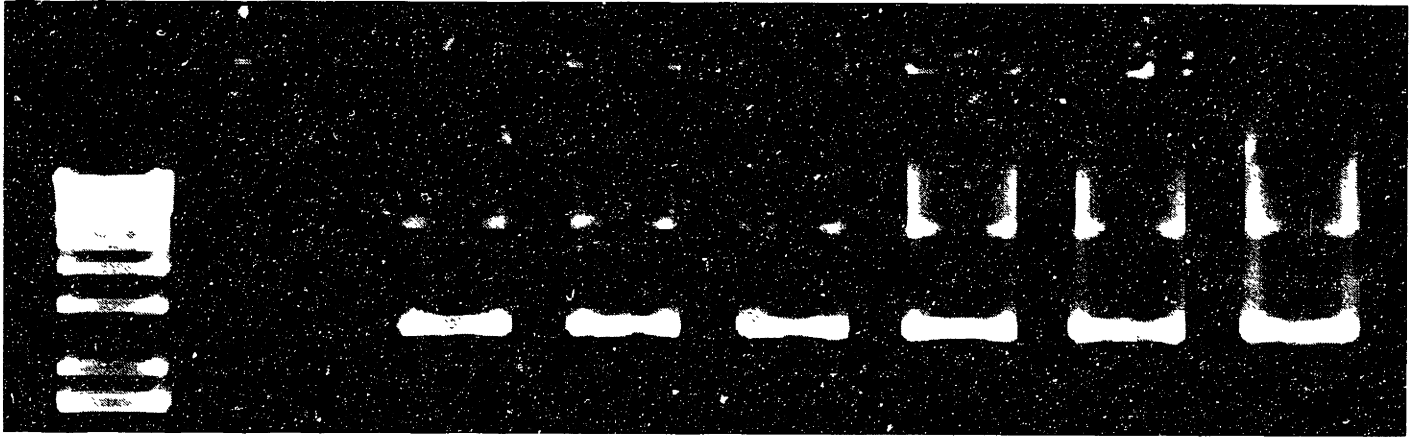
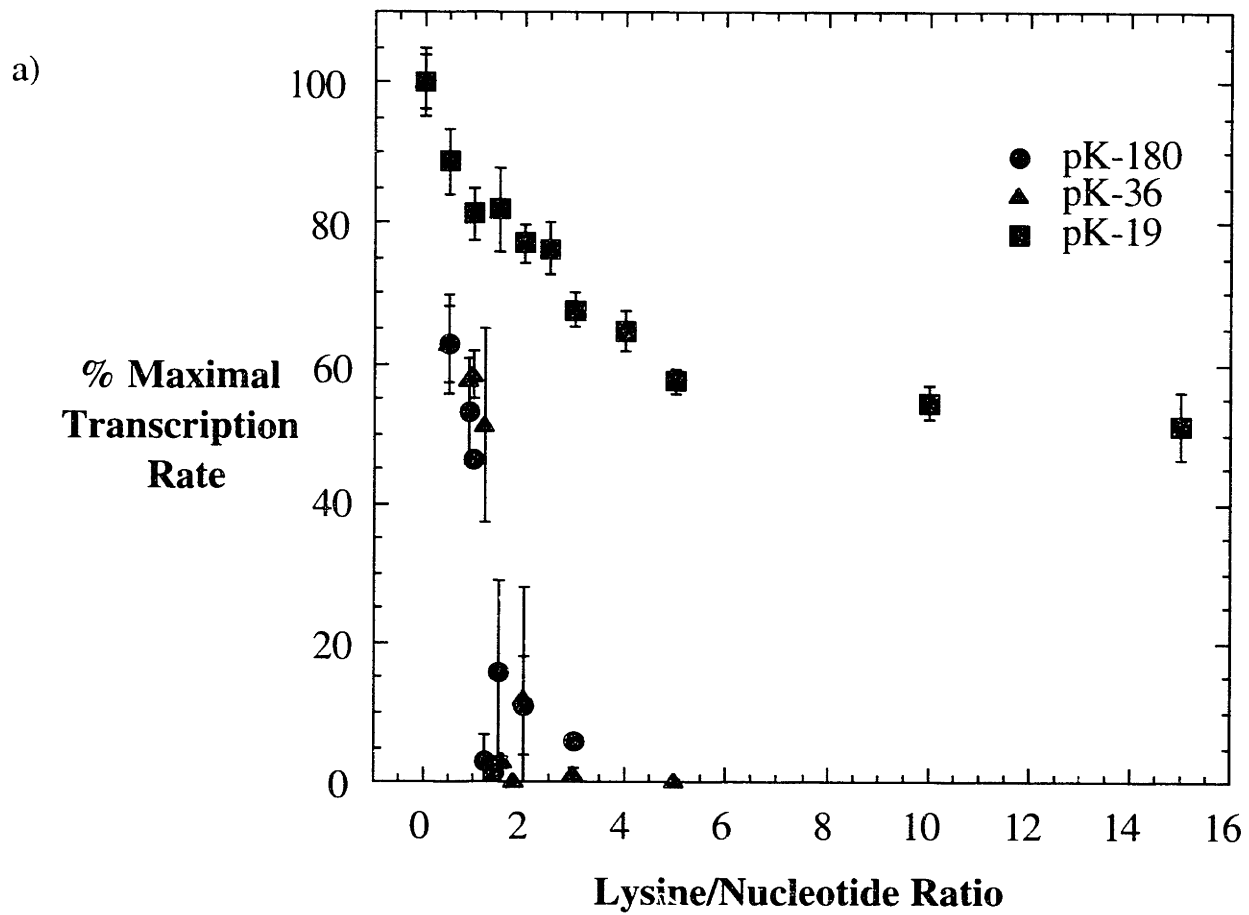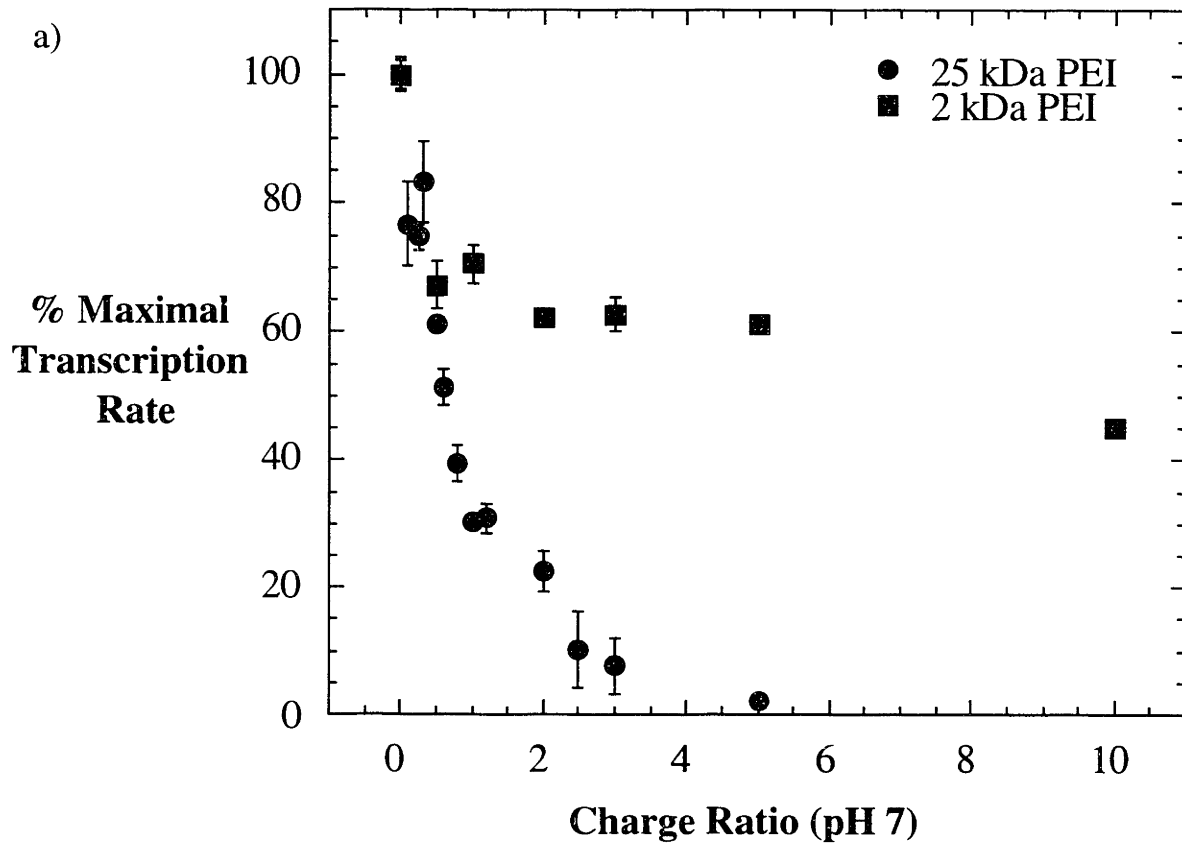
b)

c)
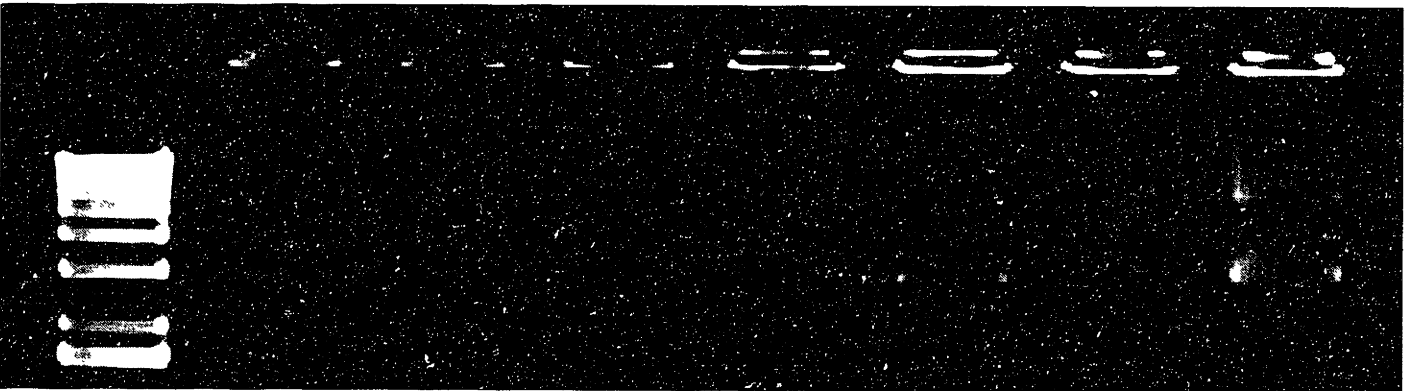
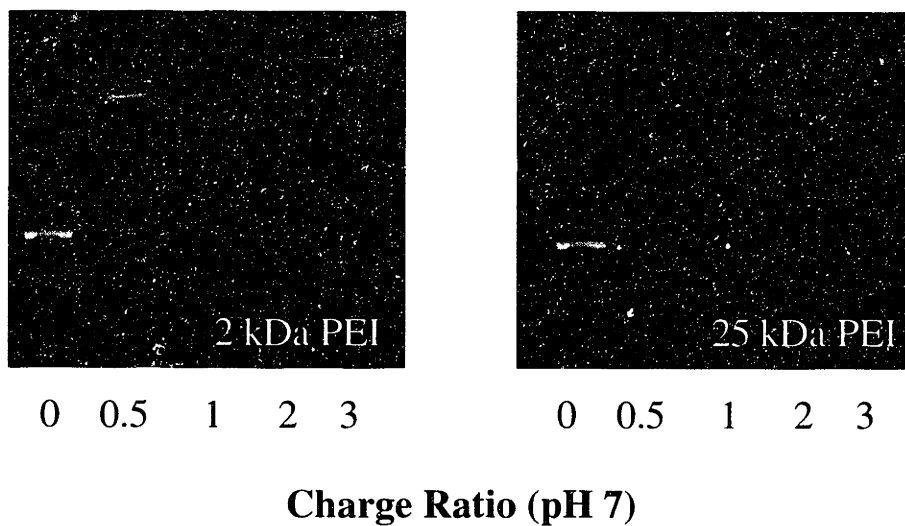
Figure 4.5 Dissociation of pK150, pK250 and pK150 from DNA
Lane 1 Standard, Lane 2 0h, Lane 3 2h, Lane 4 4h, Lane 5 12h, Lane
6 24h, Lane 7 48h, Lane 8 ish.

a)

b)
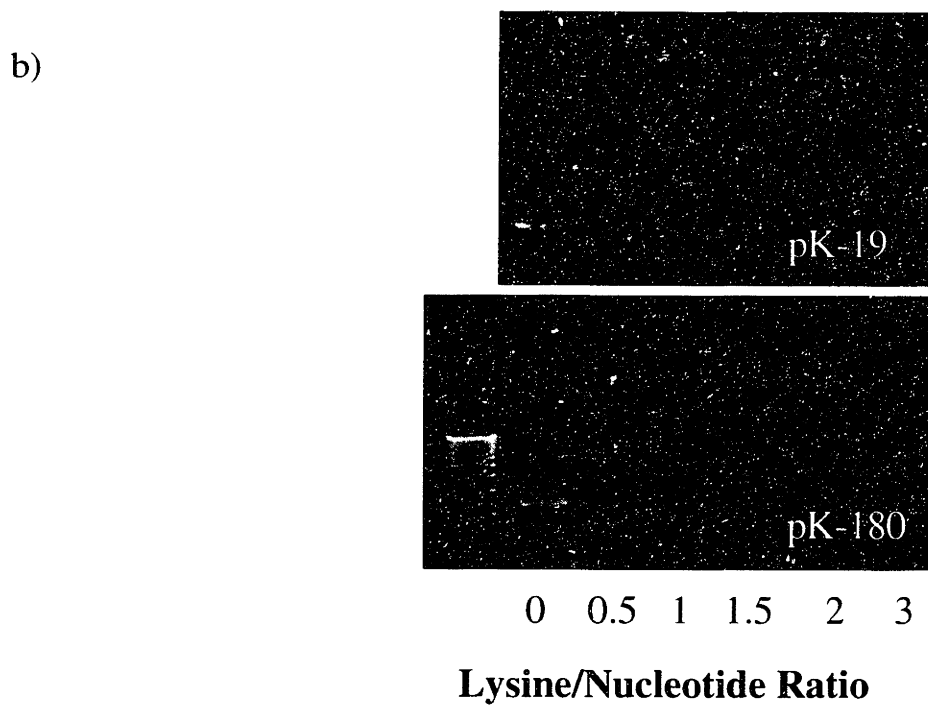
Figure 4.6 *In vitro* transcription a) inhibition by polylysine b) binding of polylysine to DNA

Figure 4.7 *In vitro* transcription a) inhibition by polyethyleneimine b) binding of polyethyleneimine to DNA

4.5 References

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5. Conclusions

If it can be successfully developed, gene therapy can provide cures for cancer, AIDS, and many genetic disorders. However, substantial progress is still needed before success in clinical trials can be realized. Improvements must be made in the maintenance and regulation of expression after delivery. However, the development of efficient gene delivery vectors also remains a significant obstacle to gene therapy.

Molecular conjugates are a vector with significant potential; however, their efficiency must be improved. To work towards this goal, we have developed a novel and versatile experimental system for molecular conjugate gene delivery. Employing this system, we have examined the mechanism of conjugate gene delivery in order to identify barriers that limit conjugate gene delivery efficiency. We subsequently found that cell surface binding and vector unpackaging are rate-limiting steps in conjugate gene delivery, the first identification of these two stages as barriers. In the process, gene delivery specificity was enhanced, and efficiency was increased from our first successful delivery to 0.5% of the cells to a final efficiency of 37%. Importantly, strategies we have identified to increase efficiency can be combined with other means, such as enhancement of endosomal escape, in order to further improve delivery. Future work will be directed at such efforts, which have been shown to relieve the dependence on chloroquine for efficienct delivery. With continued improvement, conjugates may eventually become efficient, targeted, injectable vectors.

Appendix 1   Novel Endocytic Behavior by PC12 Cells

Appendix 1.1   Background

I have a long term interest in delivering genes to cells of the central nervous system, and the most commonly used model for neurons are PC12 cells, a rat pheochromocytoma cell line (Greene et al., 1991). We therefore began to characterize the internalization of EGF by these cells as a prelude for conducting gene delivery experiments, but found a novel phenomenon in the process. This behavior was subsequently investigated for a purely scientific motivation.

Appendix 1.2   Materials and Methods

Appendix 1.2.1   Materials

Mouse EGF, penicillin-streptomycin, trypsin-EDTA, and RPMI-1640 media were obtained from Life Technologies. Mouse EGF was iodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) using Iodobeads and following the manufacturer's protocol (Pierce Chemicals). Labeled protein was separated from free iodine with a Sephadex G-25 column (Pharmacia Biotech), and its specific activity ranged from 160,000 to 220,000 cpm/ng.

Appendix 1.2.2   Cell Culture

PC12 cells, obtained from the American Type Culture Collection, were cultured in 10 cm tissue culture dishes (Corning Glass Works, Corning, NY) in RPMI 1640 medium supplemented with 5% FBS, 10% horse serum, and 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified incubator (5% CO_2) at 37°C. For experiments, cells were plated on 35 mm tissue culture plates covalently coated with polylysine to improve adhesion. Briefly, 1 ml of a filtered solution of 10 mg/ml polylysine (molecular weight 150,000-200,000) and 0.13 mg/ml carbodiimide (Aldrich Chemicals) in PBS was incubated on each 35 mm tissue culture plates. After a three hour incubation, plates were

washed twice with tissue culture grade water and used within several days. This coating was required to maintain cell adhesion during the numerous washing steps of the internalization assay.

Appendix 1.2.3   Internalization Assay

The specific endocytic rate constant was measured by the method of Wiley and Cunningham (Wiley and Cunningham, 1982). PC12 cells were seeded at approximately 1.5 million cells per plate and grown overnight. One half hour prior to the experiment, growth media was aspirated and replaced with R/H/B medium (RPMI 1640 medium with no bicarbonate, 20 mm HEPES, pH 7.4, 1 mg/ml BSA, and 1% dialyzed horse serum). ^{125}I-EGF at various concentrations in R/H/B at 37°C was added to all samples at the same time. At 1, 2, 3,4, 5, and 7 min, cells were washed three times with 3 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). Cells were then washed twice with 1 ml of acid strip (50 mM glycine, pH 3.0, 100 mM NaCl, 1 mg/ml polyvinylpyrrolidone) to remove surface-bound ligand, and the two washes were pooled for counting. Cells were then solubilized with NaOH to collect the internalized ligand. Samples were then counted with a Packard 5000 series γ-counter (Packard Instruments). To analyze the contributions of nonspecific binding of ^{125}I-EGF on the surface and internalized counts, cells were incubated with radiolabeled ligand plus 500 ng/ml EGF in some experiments.

For short periods of time, the rate of accumulation of intracellular receptor-ligand complexes C$_i$ is equal to the number of surface complexes C$_s$ multiplied by k$_e$, the specific endocytic rate constant:

$$\frac{dCi}{dt} = keCs$$

By integrating both sides of this equation between t=0 and t=t, we obtain the equation:

$$Ci = ke \int_0^t Csdt$$

From experiments we obtain C_s and C_i as a function of time, and a plot of C_i vs. the time integral of C_s should yield a line of slope k_e.

To determine the effects of serine/threonine kinases or phosphatases on EGF receptor endocytosis, PC12 cells were incubated for various times in the presence of staurosporine or okadaic acid, and internalization experiments were conducted as described above. For incubations longer than 30 min, cells were first incubated with the inhibitor in regular growth media, and then switched to inhibitor in R/H/B for the final half hour before the experiment.

Appendix 1.3   Results and Discussion

We quantified surface bound and internalized EGF as a function of time, and a sample of the data before and after integration is shown in Figure A.1.1. We conducted experiments at several ligand concentrations, and thereby varied the average surface occupancy of the EGFR during the course of the experiment. A plot of the specific endocytic rate constant as a function of the average surface complex number is shown in Figure A.2. After binding of a ligand, the tyrosine kinase within the EGF receptor tail (Figure 2.2) is activated, leading to the autophorphorylation of tyrosine sites within the regulatory region of the receptor (Sporn and Roberts, 1990; van der Geer, 1994). Active receptors are then specifically recruited to coated pits on the cell surface which bud from the cell surface during endocytosis (Mellman, 1996; Sorkin and Waters, 1993). The recruitment to the coated pits is mediated through the binding of the active receptor to protein components within the pit, and if the number of these components is comparable to the number of occupied receptors, then this process can be saturated.

As shown if Figure A.1.2, as the average C_s increases, there is a drop in the endocytic rate constant, and such saturation of endocytosis has been observed with the

EGF receptor in other systems (Starbuck et al., 1990; Wiley, 1988). However, at low C_s we also observe a rapid decrease in k_e, and this behavior has not been previously described. One possibility that could account for the phenomenon is a signal transduction feedback loop. As growth factor receptors are activated, the signal is transduced to the cell interior through the activation of a cascade of enzymes, and it has been found that some of these enzyme can in turn covalently modify the EGFR. Specifically, there is evidence that PKC, MAPK, and CAM Kinase II can phosphorylate the EGFR on serine and threonine sites. Furthermore, this phosphorylation has been shown to increase the rate of endocytosis (Countaway et al., 1992; Gamou and Shimizu, 1994; Heisermann et al., 1990). Therefore, if EGFR occupancy and resulting signal transduction levels are low, then the level of activation of these three serine/threonine kinases (S/T kinases) may be insufficient to significantly phosphorylate the EGFR. This low level of receptor phosphorylation could lead to a lower rate of endocytosis, which is what we observe in Figure 2.

Two ways to test this hypothesis are to attempt to reduce the EGFR phosphorylation on serine and threonine sites by inhibiting the S/T kinases, and to increase EGFR phosphorylation by inhibiting the phosphatases that remove the phosphate residues from the EGFR sites. We therefore tested the effects of staurosporine, a general S/T kinase inhibitor (MacKintosh and MacKintosh, 1994), on internalization at the peak of the plot shown in Figure A.1.2. Figures A.1.3 and A.1.4 show that, as predicted, staurosporine decreases k_e in a time and dose dependent fashion. Figure A.1.5 shows that after a 2 h incubation in 200 nM staurosporine, k_e undergoes a statistically significant reduction (p=0.004) from 0.31 to 0.23/min at an average C_s of approximately 2000. We next tested the effects of okadaic acid, a general S/T phosphatase inhibitor (Hernandez-Sotomayor et al., 1991; MacKintosh and MacKintosh, 1994), on endocytosis. Okadaic acid has been shown to induce the hyperphosphorylation of the EGFR and would therefore be expected to increase k_e. Figures A.1.6 and A.1.7 show that okadaic increases the endocytic rate in a

time and dose dependent fashion, and Figure A.1.8 shows a statistically significant

increase (p=0.004) in k_e from 0.17 to 0.27/min at an average C_s of approximately 60

receptors per cell after a 1 h incubation in 300 nM okadaic acid. These two pieces of data

strongly support the hypothesis that a signal transduction feedback loop can account for the

reduced k_e we observe at low C_s. Although this work is unrelated to gene delivery, it is a

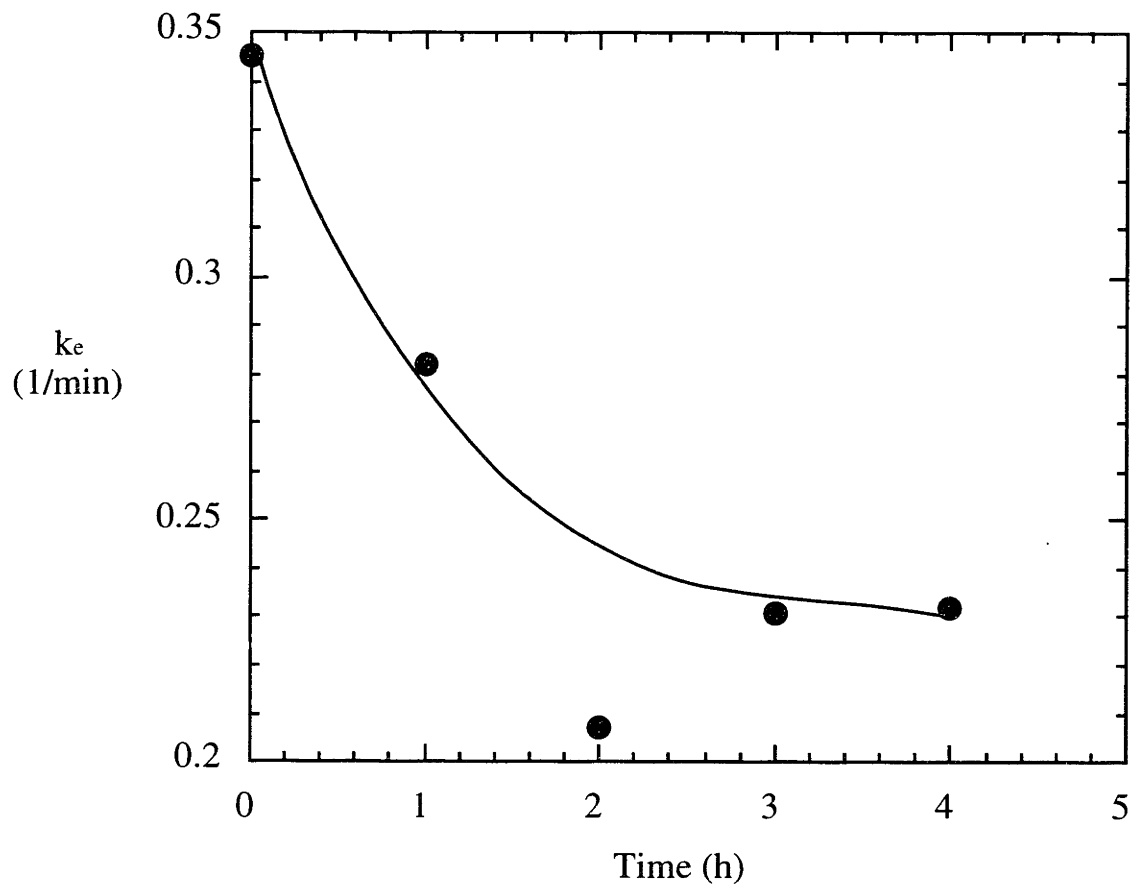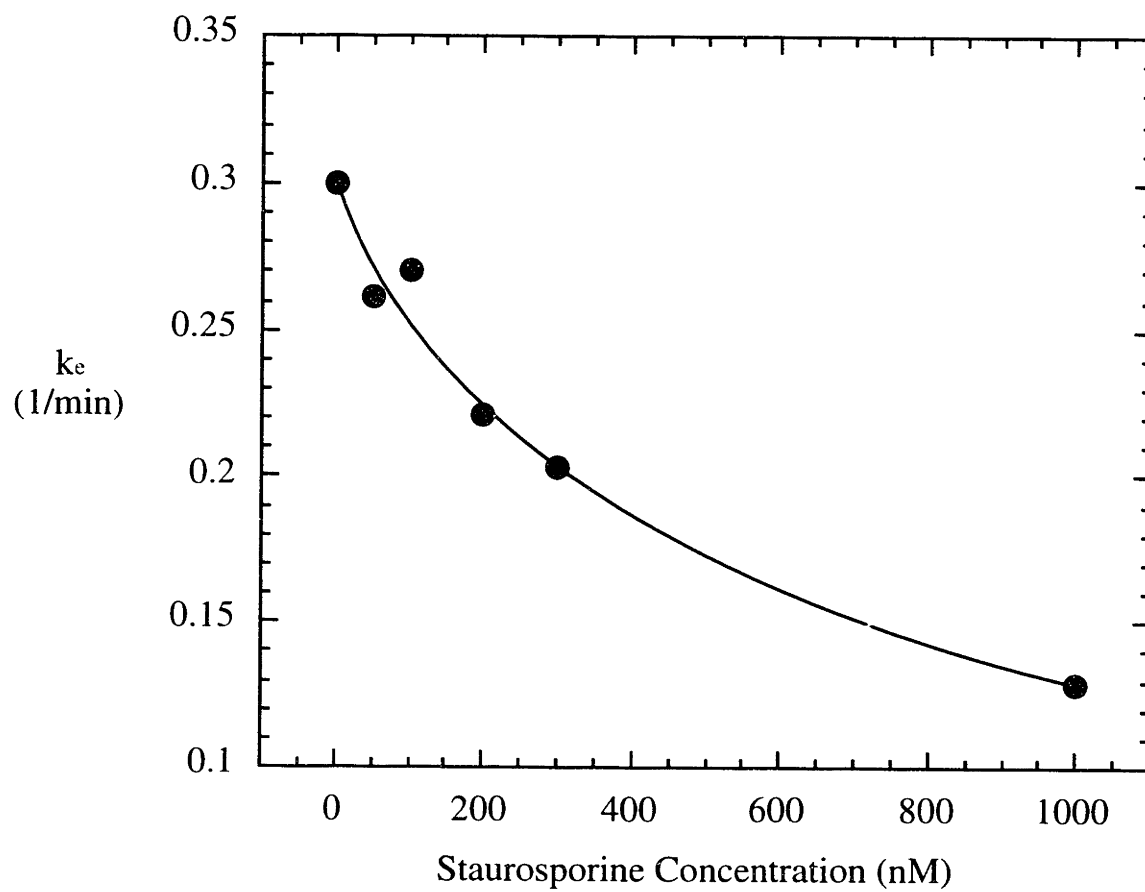novel contribution to the fields of receptor-ligand dynamics and signal transduction.
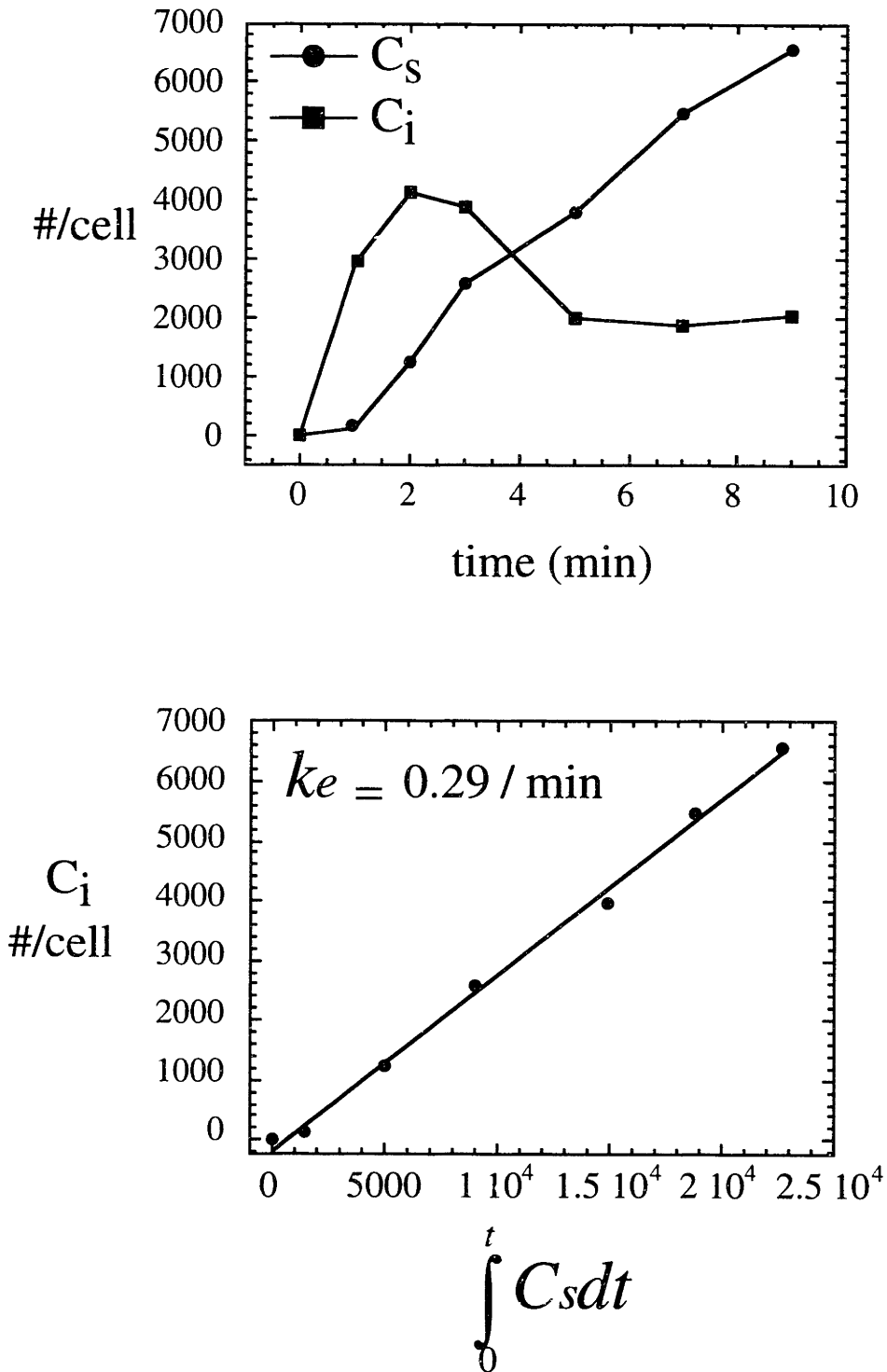
Figure A.1.1 The endocytic rate constant: a) raw data b) integrated
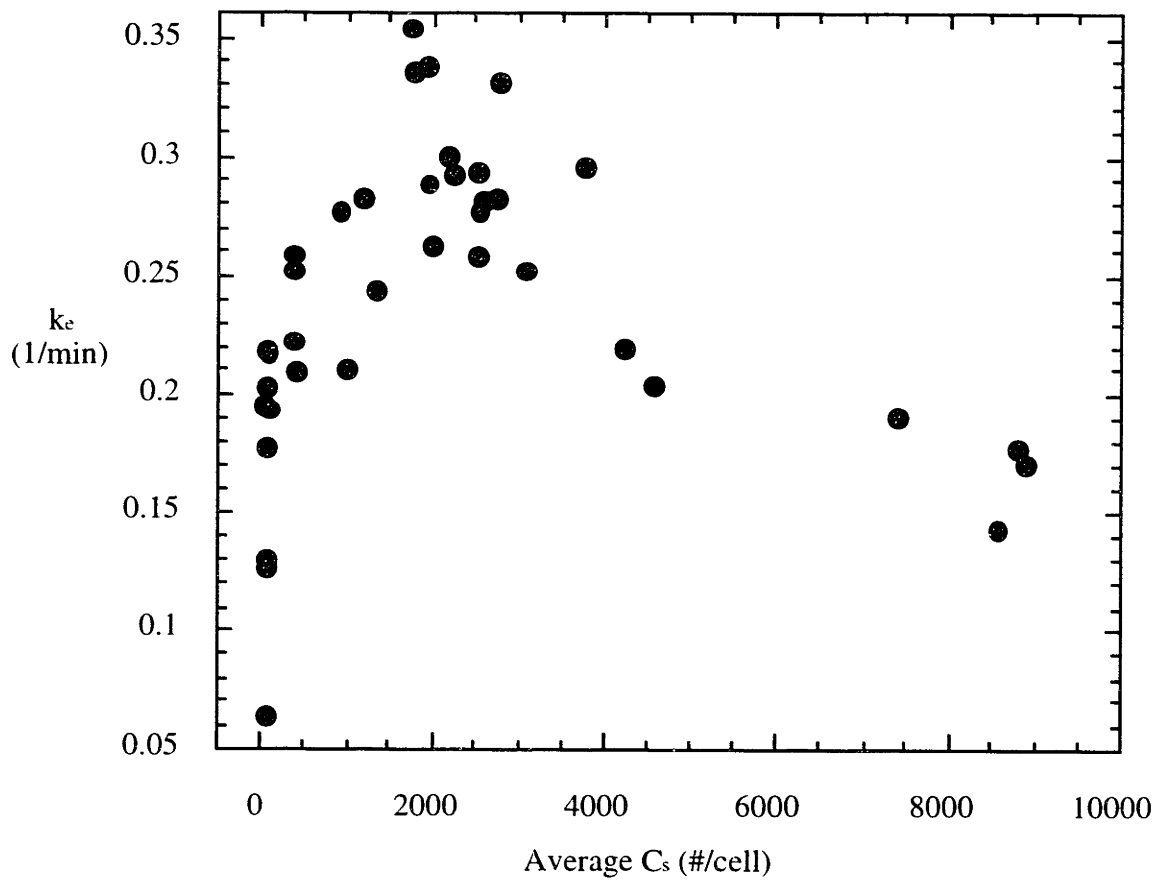
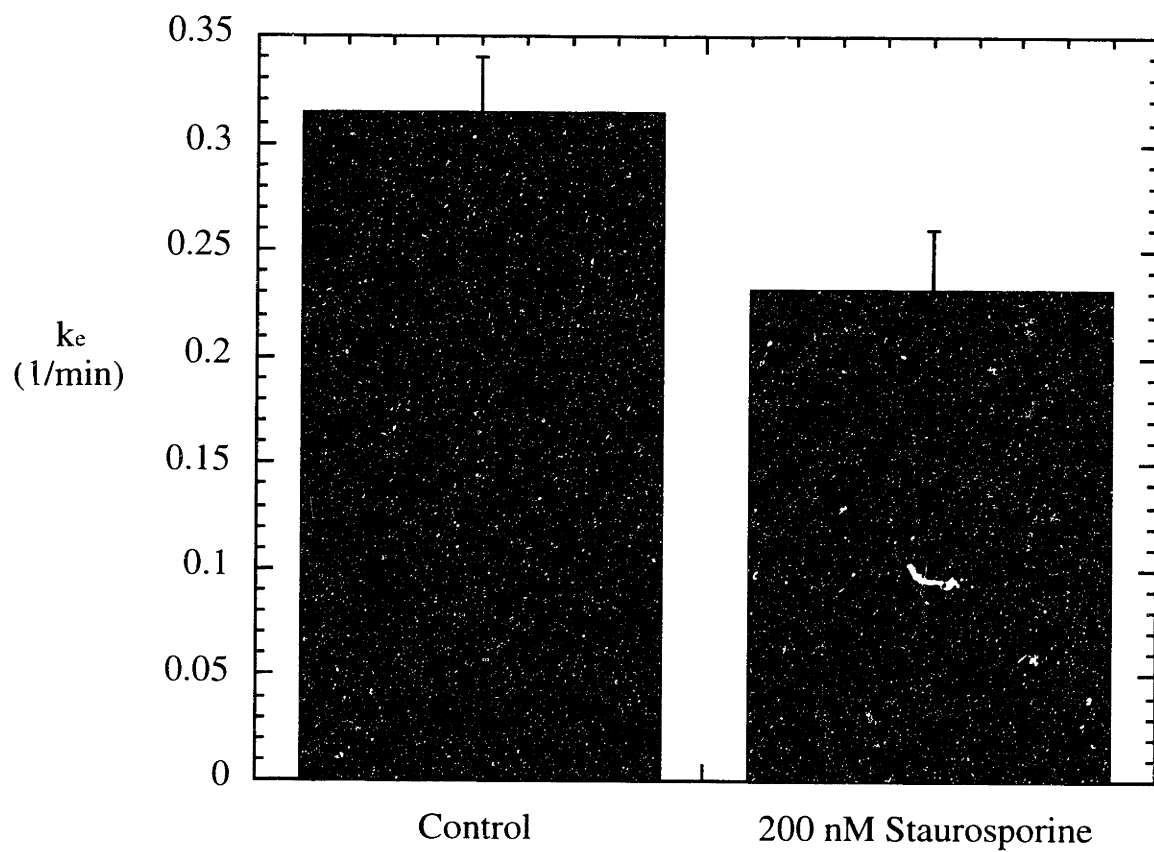**Figure A.1.2** Endocytic rate constant as a function of average EGFR occupancy

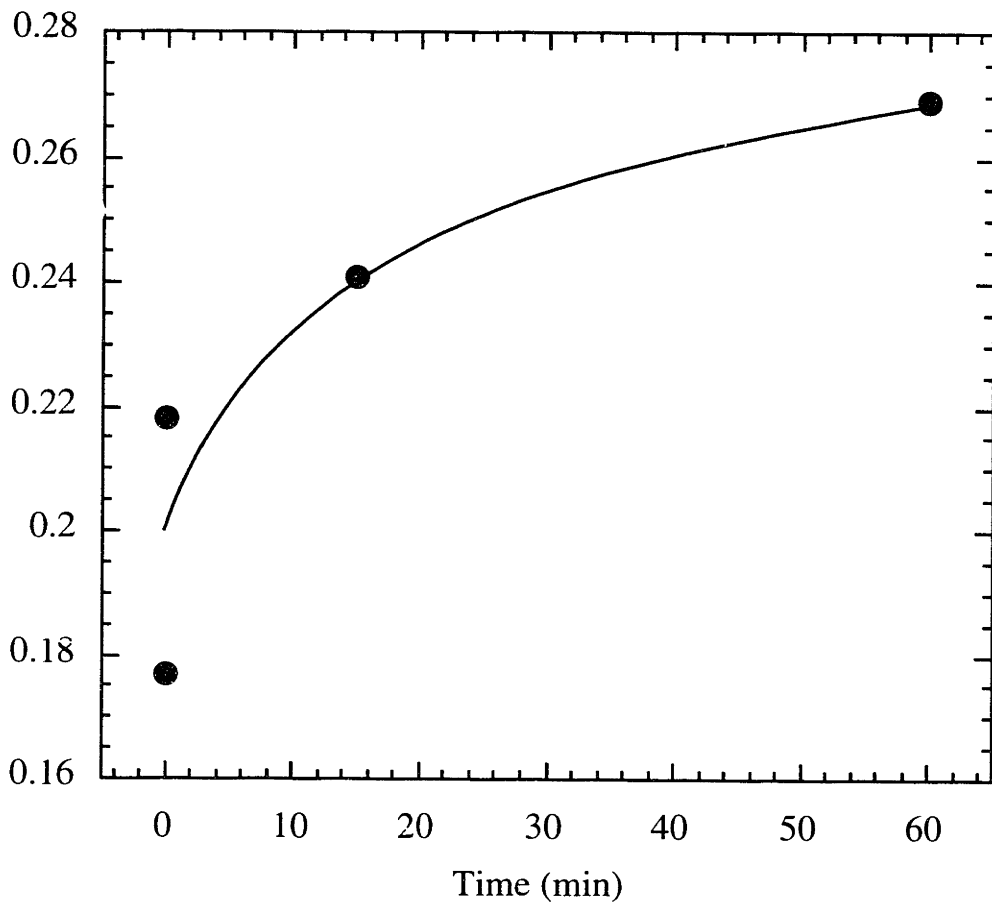**Figure A.1.3** Staurosporine reduction of k_e as a fuction of time

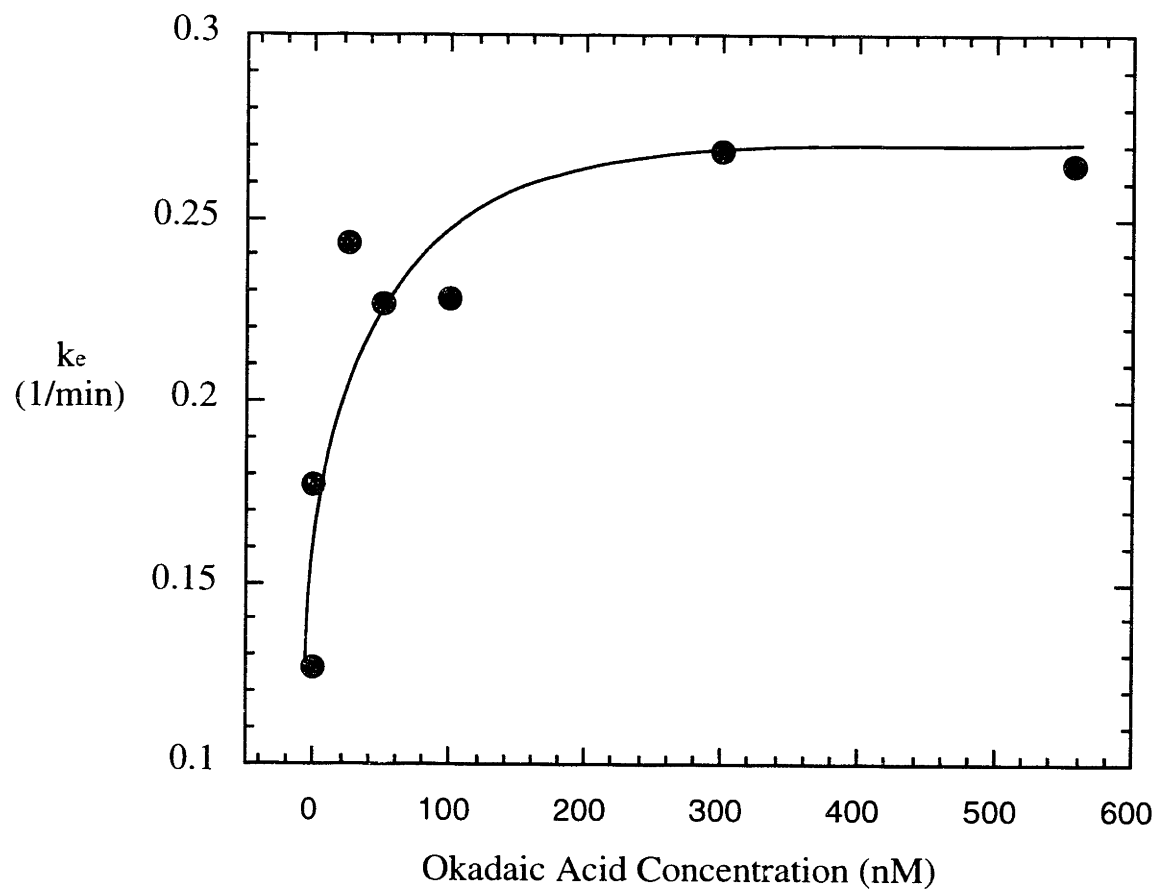**Figure A.1.4** Staurosporine reduction of k_e as a fuction of concentration
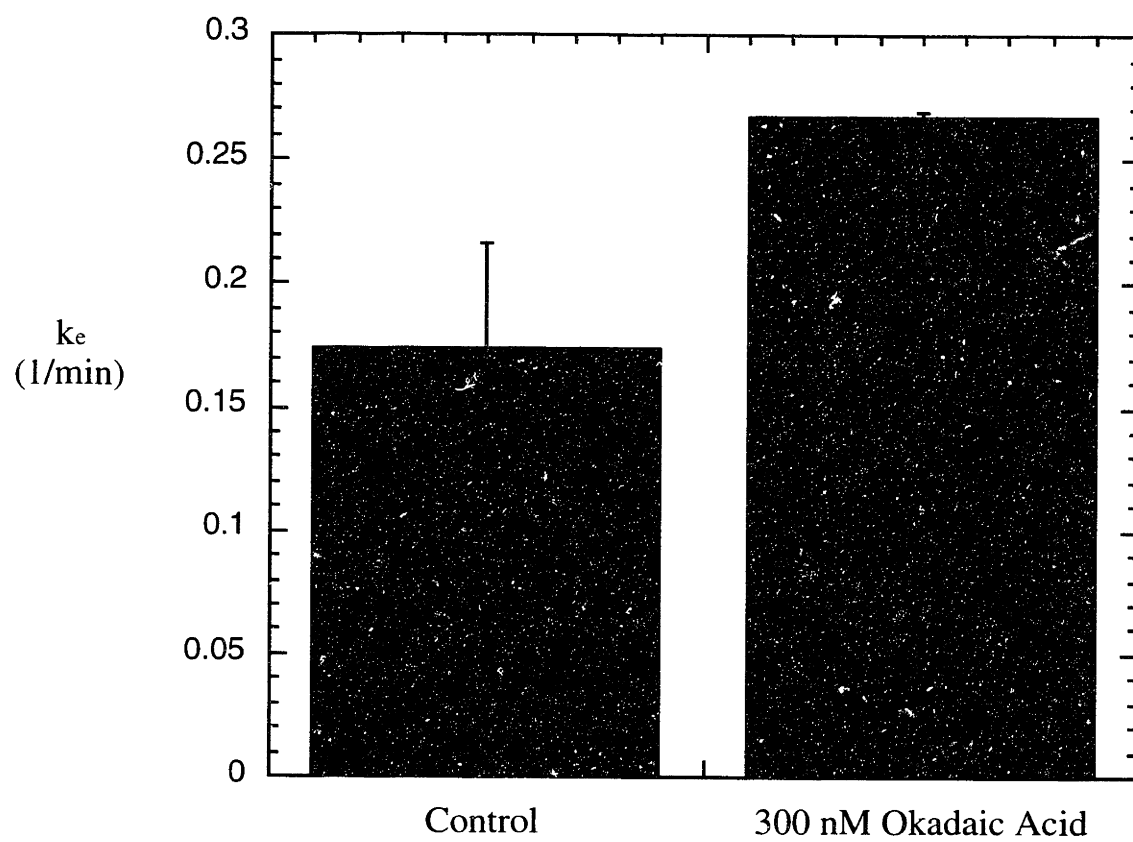
Figure A.1.5 Staurosporine reduction of k_e (errors are S.D. of n=3)

Figure A.1.6 Okadaic acid increase of k_c as a function of time

Figure A.1.7 Okadaic increase of k_e as a function of dose

Figure A.1.8 Okadaic increase of k_e (errors are S.D. of n=3)

Appendix 1.4   References

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