Interactions of liposomes and proteoliposomes with cultured cells : application to the cystic fibrosis transmembrane conductance regulator
by Carol Ann Higginbotham

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University

Abstract:
Cystic Fibrosis (CF) is the most common fatal genetic disease affecting Caucasians. Potential approaches to therapy seek to decrease or avoid disease symptoms by correction of the genetic defect. Included in these approaches are attempts to deliver a normal copy of the defective gene, and attempts to deliver normal protein, to the affected cells of CF patients.

Liposomes composed of artificial, cationic compounds have been found to be effective DNA delivery vehicles, producing expression of the gene products introduced in a significant percentage of treated cells. This study investigated the feasibility of using liposomes for delivery of integral membrane proteins, and particularly the protein defective in CF, to cultured eukaryotic cells. Because of their demonstrated usefulness in DNA transfection, cationic liposome delivery vehicles were examined in detail.

Fluorescence microscopy was used, in conjunction with fluorescence resonance energy transfer, to characterize the interaction occurring between liposomes or proteoliposomes and cultured cells. Studies of cationic liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and l,2-bis(oleoxy)-3-(trimethylammonio) propane (DOTAP), and DOPE and 3-β[N,N-dimethylaminoethane)-carbamoyl] cholesterol (DC Chol) revealed that these liposomes adhered strongly to cell surfaces, but that dilution of the probes did not occur, to a measurable degree. These results suggest that these cationic liposomes would not be suitable for integral membrane protein delivery to the cell lines studied. Other liposome formulations tested did not exhibit interaction with cultured cells.

Systems employing viral envelopes (virosomes) and receptor mediated uptake of liposomes were also studied, to see if these systems might be significantly more efficient at delivery of foreign material. In the experiments performed, no evidence of lipid transfer to target cells was obtained.

The protein defective in CF is reported to undergo rapid endocytosis from the plasma membrane. To investigate the trafficking of this protein, we identified the CFTR protein by immunoblot from vesicle enriched cell fractions. Cell fractions containing clathrin, a marker protein for coated vesicles, were found also to contain a protein of approximately 170 kD which was reactive to anti-CFTR antibodies.

INTERACTIONS OF LIPOSOMES AND PROTEOLIPOSOMES WITH CULTURED CELLS: APPLICATION TO THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

by

Carol Ann Higginbotham

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Biochemistry

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana

December 1996

APPROVAL

of a thesis submitted by

Carol Ann Higginbotham

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Martin Teintze — (Signature) — 12/12/96 (Date)

Approved for the Department of Chemistry and Biochemistry

David M. Dooley — (Signature) — 12/12/96 (Date)

Approved for the College of Graduate Studies

Robert L. Brown — (Signature) — 1/20/97 (Date)

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Signature Carol A. Huggins

Date 12/20/96

ACKNOWLEDGEMENTS

I would like to thank my academic and personal mentors who have supported me in immeasurable ways throughout the course of this study. Dr. Martin Teintze is a thorough teacher, and deserves much of the credit for the development of my abilities and this project. Other members of the academic community at Montana State University have also been particularly helpful: Sam Rogers, Ed Dratz, Mark Quinn, and Dave Dooley who have served on my graduate advisory committee, and also Cliff Bond and Bruce Granger, who have been helpful with technical issues.

Steve Swain has been a particularly important professional and personal cohort. His breadth of knowledge and true understanding of the scientific pursuit challenged and inspired me. His keen perception (in scientific and personal matters) and humor did much to keep my work interesting. His knack for knowing the right time for ice cream breaks was critical to maintaining my perspective, especially at difficult times. I owe him much.

Other coworkers have done their share, as well, to make this work possible, and enjoyable. Thanks to Daphne Moffett, Tami Peters, and Dave Parks from the Teintze group, and to Doreen Brown, Jan Rasmussen, Marcella Alvarez, Christy Ruggiero, and John Bollinger in the Dooley group. Michele McGuirl, also of the Dooley group, has been a special friend and someone who understands the added challenges of parenting while in graduate school.

Undergraduates who have fueled my conviction to teach also deserve thanks. Those who were assigned to my courses, I want to thank for inspiration and for making me aware of how little I know. Those who I have worked with on this project, I want to thank for adding enthusiasm and energy to a long and at times trying endeavor.

As an undergraduate myself, I was affected profoundly by some fabulous teachers: Dave Erkenbrack, Cathy Haustein, Dan Bruss, Art Bosch, and John Bowles, none of whom ever doubted my ability to reach my goals, and gave me the courage to try.

Most influential, of course, is my family. My extended family has never questioned my ambition, they have expressed their pride, and I am grateful. My husband David has endured hardships because of my desire to complete an advanced degree, without complaints. He is a wonderfully supportive partner. And finally, there is Patrick, who thinks by now that school is a lifelong endeavor. I hope what I have done will benefit him greatly.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

TABLE OF CONTENTS--Continued

TABLE OF CONTENTS--Continued

LIST OF FIGURES

LIST OF FIGURES--continued

LIST OF FIGURES--Continued

ABSTRACT

Cystic Fibrosis (CF) is the most common fatal genetic disease affecting caucasians. Potential approaches to therapy seek to decrease or avoid disease symptoms by correction of the genetic defect. Included in these approaches are attempts to deliver a normal copy of the defective gene, and attempts to deliver normal protein, to the affected cells of CF patients.

Liposomes composed of artificial, cationic compounds have been found to be effective DNA delivery vehicles, producing expression of the gene products introduced in a significant percentage of treated cells. This study investigated the feasibility of using liposomes for delivery of integral membrane proteins, and particularly the protein defective in CF, to cultured eukaryotic cells. Because of their demonstrated usefulness in DNA transfection, cationic liposome delivery vehicles were examined in detail.

Fluorescence microscopy was used, in conjunction with fluorescence resonance energy transfer, to characterize the interaction occurring between liposomes or proteoliposomes and cultured cells. Studies of cationic liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and 1,2-bis(oleoxy)-3-(trimethylammonio) propane (DOTAP), and DOPE and 3-β-[N,N-dimethylaminoethane)-carbamoyl] cholesterol (DC Chol) revealed that these liposomes adhered strongly to cell surfaces, but that dilution of the probes did not occur, to a measurable degree. These results suggest that these cationic liposomes would not be suitable for integral membrane protein delivery to the cell lines studied. Other liposome formulations tested did not exhibit interaction with cultured cells.

Systems employing viral envelopes (virosomes) and receptor mediated uptake of liposomes were also studied, to see if these systems might be significantly more efficient at delivery of foreign material. In the experiments performed, no evidence of lipid transfer to target cells was obtained.

The protein defective in CF is reported to undergo rapid endocytosis from the plasma membrane. To investigate the trafficking of this protein, we identified the CFTR protein by immunoblot from vesicle enriched cell fractions. Cell fractions containing clathrin, a marker protein for coated vesicles, were found also to contain a protein of approximately 170 kD which was reactive to anti-CFTR antibodies.

CHAPTER 1

INTRODUCTION

Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive genetic disease affecting one in 2500 caucasians [1]. There is no cure, and progressive loss of function in affected organs generally leads to death before middle age. The current median age of death from CF is 29, however recent improvements in treatment are causing the median age of death to rise. Early and aggressive treatment, general quality of health, and the particular genetic defect an individual carries all affect the age of mortality.

Cystic Fibrosis was first described in the medical literature in the late 1930s. The syndrome includes pancreatic exocrine insufficiency, male infertility, and recurring infection of the lung leading to development of inelastic scar tissue, or "fibrosis," in the lungs. An elevated sweat chloride level in affected individuals is diagnostic of the disease [1].

Pancreatic insufficiency can be effectively treated by enzyme replacement, and most morbidity involves symptoms affecting the lungs. CF patients have difficulty clearing mucoid secretions from the lungs. It is suspected that the associated difficulty in removing bacteria increases susceptibility to colonization and chronic infection [1]. Changes in bacterial

adhesion and impaired ability of the body to kill the bacteria have also been implicated in complications in the lungs [2-5]. Damage caused by colonizing bacteria and subsequent inflammation results in the development of inelastic scar tissue and progressively declining lung function.

The metabolic disorder causing the symptoms of Cystic Fibrosis was not well understood until the genetic defect responsible was identified in 1989. DNA polymorphisms were used to map the locus of the genetic defect to a region on chromosome 7. Genetic analysis of diseased individuals and DNA analysis of a sequence thought to contain the CF locus revealed the location of the gene. The cDNA identified aligns with an open reading frame, with transcription patterns that correlate with the tissues that are affected in the disease [6-8].

The gene encodes an intregal membrane protein of 1480 residues termed the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), with a predicted molecular weight of 168 kilodaltons [6]. The predicted structure contains a pair of similar motifs, with each domain containing six transmembrane helices and a nucleotide binding fold. There is a single regulatory domain on the cytoplasmic side of the protein, containing sites amenable to regulation by phosphorylation by cAMP-dependent protein kinase [9]. The protein is glycosylated on the extracellular surface, with the sugars contributing about 30 kilodaltons to the apparent molecular weight as indicated on polyacrylamide gels [10].

Studies of purified recombinant CFTR indicated that the protein is a regulated ion channel, with properties consistent with the specific activities of cAMP regulated chloride channels in cell types that normally express

CFTR [8]. However, discussion continues over its potential activity as a regulator of other cellular functions [11-15]. Glycosylation apparently does not affect activity [16].

In 1991, it was discovered that introduction of a normal copy of the gene restores chloride channel activity in *cf* - /- cells isolated from an affected individual. Drumm et al. used retroviral delivery of a normal copy of the gene to a cell line derived from a CF-patient, to demonstrate correction of the defect [17]. cAMP stimulated ion efflux and patch clamp experiments revealed the correction of the defect by introduction of *cftr* DNA. Rich et al., in a concurrently published paper, utilized a fluorescent method to trace ion efflux, and also patch clamping, to attain the same result in cultured CF airway epithelial cells. Additionally, they demonstrated that expression of a gene carrying a common mutation did not correct the defect [18]. The connection between CFTR and ion transport dysfunction was made clear.

Sequence analysis has revealed that approximately 70% of all diagnosed individuals contain a *cftr* gene missing 3 base pairs, resulting in deletion of a phenylalanine at residue 508 in the mature protein (ΔF508). The remaining 30% of CF cases involve any one of over 200 different faults in the gene that have been identified [19].

A wide disparity in disease severity exists among the other 200+ identified mutations existing. Relationships between the effect of a particular mutation on the protein and severity are unclear [20]. Some individuals with particularly mild forms of CF can even go undiagnosed until early adulthood [21]. Environmental factors, the existence of multiple

mutations on one copy of the gene or heterozygous mutants, and the possibility that other genetic factors play a role in disease presentation, all obscure the correlation [20].

Mutations affecting protein production can be classified into four types [19]. Firstly, nonsense mutations, frameshift, or splicing errors can reduce or eliminate protein production. Secondly, mutations affecting protein processing can lead to decreased amounts of protein at the apical cell surfaces where it is normally expressed. Thirdly, some mutations cause defects in regulation of the protein. Finally, there are mutations which affect the ion conductance ability of the protein. Delta F508, which is by far the most common mutation, is a defect at the processing level. The deletion occurs within the first nucleotide binding fold (see figure 1). The CFTR protein is transcribed and translated normally, but accumulates, incompletely glycosylated, in the endoplasmic reticulum [23,24]. It is eventually degraded [22].

The reason why this form of the protein does not reach the plasma membrane is not understood. The most likely explanation is that the mutation causes improper folding of the protein, stimulating degradation of the protein from the ER, rather than proper recognition by transport machinery which should direct it to the cell surface. Interestingly, in cells grown at reduced temperature, the ΔF508 mutant form of CFTR is delivered in fully glycosylated, and at least somewhat functional, form to the cell membrane [8,25].

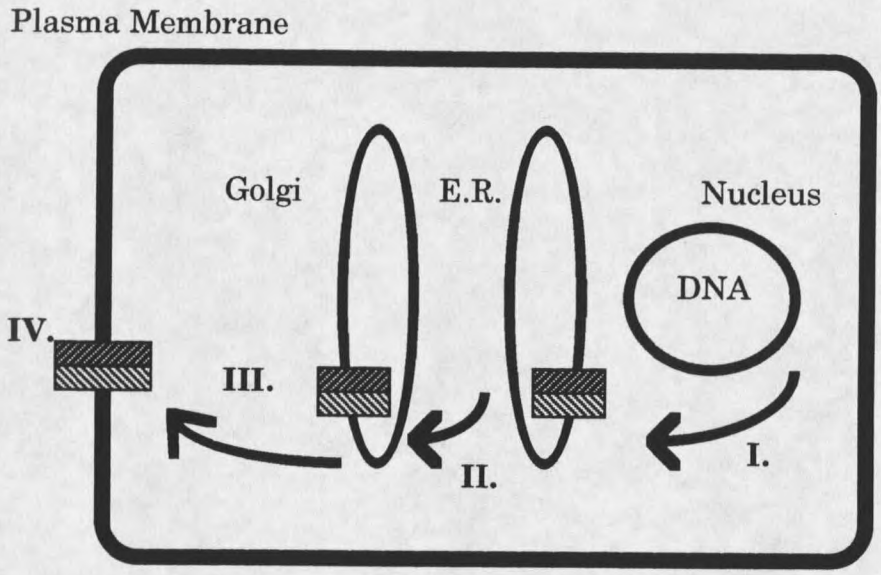

Figure 1: What goes wrong in cftr mutations causing cystic fibrosis. Four types of mutation altered membrane potential: I. Defective protein production, II. Defective protein processing, III. Defective regulation, and IV. Defective ion conduction

The resultant lack of protein at the apical surface of bronchiolar epithelium is proposed to cause an imbalance in ion transport at that surface. Compensatory changes in sodium channel activity compound the problem by altering water balance across the cell membrane [26]. The viscosity of the mucoid secretions in the channels of the glands where CFTR is most actively expressed is thought to increase due to the osmotic effect.

The CFTR

Sequence analysis predicts the normal protein to be composed of two very similar motifs, plus a large regulatory domain. Structural characteristics of each motif are very similar, although sequence homology between them is modest. Each motif contains six putative transmembrane helices, for a total of twelve spans of the membrane, presumably forming a channel. Each motif also contains a predicted nucleotide binding fold of at least 150 amino acids.

Little of the protein is extracellular, although there are two extracellular N-linked glycosylation sites. The cytosolic surface of the protein carries the two hydrophilic nucleotide binding sites, and the highly charged regulatory domain.

It is thought that phosphorylation at several sites may stimulate opening and closing of the membrane channel through conformational changes at the regulatory domain [6] (see figure 2).

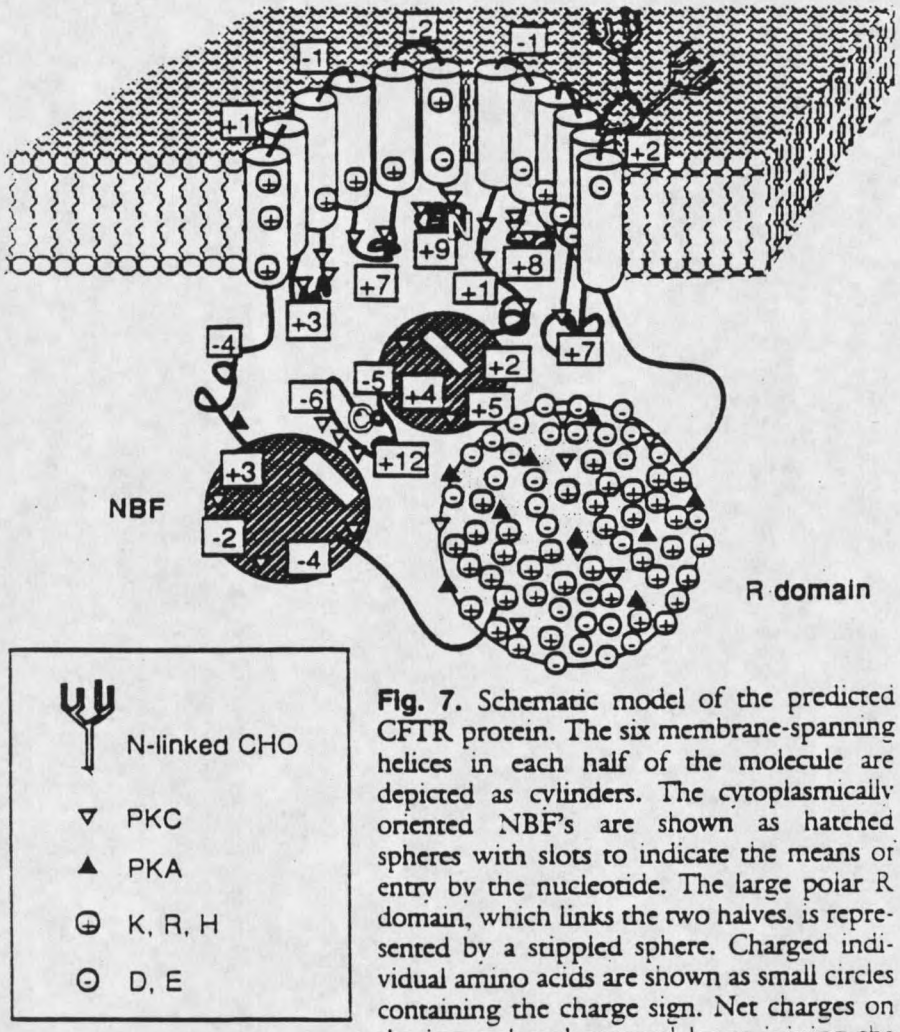

Fig. 7. Schematic model of the predicted CFTR protein. The six membrane-spanning helices in each half of the molecule are depicted as cylinders. The cytoplasmically oriented NBF's are shown as hatched spheres with slots to indicate the means of entry by the nucleotide. The large polar R domain, which links the two halves, is represented by a stippled sphere. Charged individual amino acids are shown as small circles containing the charge sign. Net charges on the internal and external loops joining the membrane cylinders and on regions of the NBF's are contained in open squares. Potential sites for phosphorylation by protein kinases A or C (PKA or PKC) and N-glycosylation (N-linked CHO) are as indicated. K, Lys; R, Arg; H, His; D, Asp; and E, Glu.

Figure 2: A schematic model of the CFTR protein, as proposed from analysis of amino acid sequence; reprinted from Riordan, et al., Science 245: 1066-1073.

CFTR belongs to a family of proteins called the ATP binding cassette proteins, or the ABC family. Included in this family are several other transmembrane transporters, notably the multidrug resistance protein, or P-glycoprotein, proposed to be responsible for drug efflux from cells resistant to chemotherapeutics [27,28].

Debate about CFTR's function has continued since its original characterization. As the name implies, CFTR has been considered to be a regulator, as well as an ion channel itself. The low copy number of the expressed protein, and the fact that other chloride channels exist in cells containing CFTR, suggest that CFTR may have functions outside of its role as chloride channel. In particular, a relationship between CFTR and the outwardly rectifying chloride channel, or ORCC, may exist [27,30]. It has also been proposed that CFTR acts as an ATP pump, driving nucleotides to the extracellular milieu [12].

Emerging Approaches to Therapy

Current therapeutic approaches to treating the pulmonary symptoms of Cystic Fibrosis are aimed at aggressively treating bacterial colonization and improving the ability to clear lung secretions. Physical elimination of the secretions is aided by chest and back percussion therapy. Enzymatic therapies for decreasing sputum viscosity have been recently introduced. Recombinant DNase has been used with some success to destroy viscous DNA deposits in the lung resulting from inflammation [31]. Improvement in the ion balance, and therefore water balance, of cellular

secretions has been attempted by blocking sodium channels in the lungs with the compound amiloride [32]. All of these methods have contributed to a noticeable increase in life expectancy for patients, but further developments in therapy will be necessary before those stricken with CF can enjoy a full life.

A goal of the CF research community is to develop therapies which more directly affect the molecular deficiency caused by the disease. Correction of the defect at the molecular level would be the most efficient kind of therapy, making it possibile to avoid the escalation of pulmonary damage caused by repeated infections.

Of particular interest are investigations into the use of gene transfer for correction of the genetic defect. Proponents argue that by introducing a normal copy of the *cftr* gene into the cells of an affected person, they can stimulate production of normal protein and alleviate symptoms. Delivery of the *cftr* gene has been shown to be possible *in vitro* and in the lungs of mouse models and cotton rats [33-35], but has yet to be shown adequate to relieve CF-affected individuals. CFTR gene transfer studies have been ongoing for several years, without reports of success. The effects of ongoing treatment with such therapies are unknown at this time.

Specifically, issues surrounding the immunogenicity and safety of gene delivery therapies are unresolved [36]. Clinical trials using liposomal vectors and adenoviral vectors are currently underway [37-40]. Both methods produce some expression, but expression levels may be inadequate and tend to be patchy across a tissue. Expression is in all cases transient, and therapies for gene delivery will need to be administered repeatedly [41].

This fact raises questions regarding the involvement of the immune response, and potential decreases in effectiveness over time. The most likely scenario is that even after development of gene therapies, a combination of clinical approaches will be used to combat the disease.

Alternative approaches to restore normal ion conductance include attempts at manipulating regulation and trafficking of CFTR, and upregulating the activity of the protein at the plasma membrane. In addition, CFTR's potential function as a regulator, rather than a significant ion conductor itself, may open doors to new approaches aimed at correcting the metabolic defect further down the line; i.e. by finding ways of changing regulation of proteins normally regulated by CFTR.

Endocytosis, Recycling, and CFTR

Certain plasma membrane proteins are known to be transported through endosomal pathways and back to the plasma membrane. This process, termed recycling, occurs through clathrin coated vesicle endocytosis, but the return of the protein to the cell membrane involves unidentified vesicles. The process is selective, involving only certain components of the plasma membrane. CFTR has been reported to cycle through such a pathway, although why CFTR would participate in such a pathway is not known [42,43].

Studies of vesicle trafficking are difficult because they rely on the identification of marker proteins specific to a particular type of vesicle in order to characterize that subpopulation of vesicles. In some instances, there are no vesicle-type specific markers [44,45]. Physical identifiers such

as size and density are somewhat characteristic of a vesicle subpopulation, although they generally are not adequately distinctive to separate vesicle populations unequivocally [46].

Studies of yeast secretory mutants have led to a fairly thorough understanding of the mechanisms involved in vesicle traffic from the endoplasmic reticulum to the plasma membrane. In all instances, membrane trafficking along this route is carefully controlled by ligand-receptor interactions which stimulate fusion of vesicle membranes [47-49]. Identification of the ligands and receptors involved, mutation studies,and immunocytochemistry allow for detailed studies of these systems.

Similar model systems do not exist for studying recycling. The existence of proteins regulating the endocytosis selection system, and subsequent sorting of vesicle components and downstream routing, is undetermined. Studies rely, rather, on compounds thought to inhibit endocytosis, such as brefeldin A [50], or the vesicle acidification processes, such as chloroquine [51]. Because of the general lack of understanding about the endocytosis/recycling pathway, these studies are inadequate to draw conclusions about how sorting and trafficking in the recycling pathway occurs.

What is known is that certain identifiable proteins are selectively drawn into the recycling pathway, and that among those proteins is the CFTR. CFTR is functional in endosomes, but does not appear to affect the acidification or regulation of the endocytic system [53,54]. It is plausible that CFTR, like most membrane proteins in endosomes, gets recycled to the plasma membrane [54-56].

Vesicles containing compounds bound for endocytosis get internalized through either coated or noncoated pits. Coated pits, the usual route for internalization of receptor-ligand complexes, are so called because they are surrounded by a protein coat at the cytosolic face of the membrane. The fibrous coat protein clathrin assembles at the site of membrane invagination as the vesicle buds and dissociates from the vesicle after internalization. Sometime after dissociation of the clathrin from the vesicle, sorting occurs and certain components of the vesicle membrane are routed back to the plasma membrane [45].

The internal pH of endosomes varies from near physiological pH to as low as 5.0. Separation of ligands from their receptors during endocytosis and recycling is controlled by the endosomal pH, so that as the pH is reduced, the ligand dissociates and is free to be trafficked separately from the receptor [57]. This process provides a way for the cell to sort ligands from the extracellular milieu to the lysosome for degradation, while recycling the receptor.

Delivery Systems

Cell membranes act as selective barriers to most substances. Overcoming this barrier is necessary for efficient delivery of any foreign molecule, be it DNA, protein, or other compounds, to the cell. The development of delivery vehicles, or vectors, with adequate efficiency to cause physiological changes in tissues has proven difficult. In the arena of DNA transfection, dramatic improvements have come in the last ten