

# Uptake Pathways and Subsequent Intracellular Trafficking in Nonviral Gene Delivery

IKRAMY A. KHALIL, KENTARO KOGURE, HIDETAKA AKITA, AND HIDEYOSHI HARASHIMA

Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku, Sapporo, Hokkaido, Japan (I.A.K., K.K., H.A., H.H.); and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Japan (I.A.K., K.K., H.A., H.H.)

Abstract .....	32
I. Introduction .....	33
II. Uptake pathways for nonviral gene delivery .....	33
A. Endocytic uptake pathways .....	34
1. Clathrin-mediated endocytosis .....	34
2. Caveolae-mediated endocytosis .....	35
3. Macropinocytosis .....	36
4. Phagocytosis .....	37
5. Receptor-mediated endocytosis .....	37
B. Nonendocytic delivery .....	38
III. Tools to study intracellular trafficking in nonviral gene delivery .....	38
A. Perturbation of the endocytosis-mediated uptake and intracellular trafficking .....	38
B. Quantitative evaluation of intracellular trafficking .....	39
IV. Uptake mechanisms and intracellular trafficking of gene delivery mediated by cationic lipids and polymers .....	40
A. Interaction between DNA and cationic lipids or polymers .....	40
B. Cellular binding .....	41
C. Cellular uptake .....	41
D. Endosomal escape .....	42
E. Nuclear delivery .....	43
V. Conclusions .....	43
Acknowledgments .....	44
References .....	44

**Abstract**—The successful delivery of therapeutic genes to the designated target cells and their availability at the intracellular site of action are crucial requirements for successful gene therapy. Nonviral gene delivery is currently a subject of increasing attention because of its relative safety and simplicity of use; however, its use is still far from being ideal because of its comparatively low efficiency. Most of the currently available nonviral gene vectors rely on two main components, cationic lipids and cationic polymers, and a variety of functional devices can be added to further optimize the systems. The design of these functional devices depends mainly on our understanding of the mechanisms involved in the cellular uptake and intracellular disposition

of the therapeutic genes as well as their carriers. Macromolecules are internalized into cells by a variety of mechanisms, and their intracellular fate is usually linked to the entry mechanism. Therefore, the successful design of a nonviral gene delivery system requires a deep understanding of gene/carrier interactions as well as the mechanisms involved in the interaction of the systems with the target cells. In this article, we review the different uptake pathways that are involved in nonviral gene delivery from a gene delivery point of view. In addition, available knowledge concerning cellular entry and the intracellular trafficking of cationic lipid-DNA complexes (lipoplexes) and cationic polymer-DNA complexes (polyplexes) is summarized.

Address correspondence to: Dr. Hideyoshi Harashima, Laboratory for Molecular Design of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6, Sapporo City, Hokkaido 060-0812, Japan. E-mail: harasima@pharm.hokudai.ac.jp

This work was supported in part by grants-in-aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by grants-in-aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science.

Article, publication date, and citation information can be found at <http://pharmrev.aspetjournals.org>.

doi:10.1124/pr.58.1.8.

**I. Introduction**

Gene therapy is a new therapeutic strategy that offers the promise of treating diseases via the production of therapeutic proteins within cells. In general, any drug molecule must reach its intended site of action to exert its therapeutic effect. If this does not occur, the drug will have no therapeutic activity and may even cause non-specific effects through interactions with nonintended targets. The problem of drug delivery is even more complicated when large, charged molecules such as DNA are used as drugs, as is the case in gene therapy. For nucleic acid molecules that are used in gene therapy, the target sites are mostly inside the cells, in the cytoplasm or the nucleus. Therefore, it is essential that these molecules traverse the plasma membrane to reach their target sites (Bally et al., 1999). The plasma membrane of living cells is a dynamic structure that is relatively lipophilic in nature. As a result, it restricts the entry of large, hydrophilic, or charged molecules. Most genetic molecules are both large and charged, making it difficult for them to traverse the plasma membrane on their own, and an appropriate gene delivery system is therefore required for their efficient cellular uptake. Synthetic or nonviral gene delivery systems can circumvent some of the problems associated with viral vectors such as non-specific inflammations and an unexpected immune response (Nabel et al., 1993; Smith et al., 1993b; Yang et al., 1994). Furthermore, nonviral vectors have advantages in terms of simplicity of use and ease of large-scale production. However, the comparatively low efficiency is a main disadvantage of nonviral vectors, and efforts are ongoing to increase their efficiency (Li and Huang, 2000).

Several biological barriers must be overcome to achieve efficient nonviral gene delivery (Bally et al., 1999). These barriers include binding to the cell surface, traversing the plasma membrane, escaping lysosomal degradation, and overcoming the nuclear envelope. The most common materials used in current nonviral preparations include lipids (typically a mixture of cationic and neutral lipids), cationic polymers, peptides, or combinations thereof (Wu and Wu, 1987; Smith et al., 1993a; Ledley, 1994; Boussif et al., 1995; Lee and Huang, 1996; Futaki et al., 2001; Torchilin et al., 2003; Khalil et al., 2004). Functional devices can be further introduced to overcome different cellular barriers and include the use of targeting ligands to increase cellular uptake through receptor-mediated endocytosis, membrane active lipids and peptides to enhance the endosomal release, and nuclear localization signals to enhance nuclear delivery (Wagner et al., 1992; Plank et al., 1994; Sebestyen et al., 1998; Tachibana et al., 1998; Simoes et al., 1999). The appropriate design of a nonviral gene vector requires a complete understanding of both the characteristics of the vectors as well as the mechanisms by which they interact with the targeted cells. Several internalization

mechanisms have been proposed to explain the uptake of different synthetic vectors (Felgner et al., 1987, 1995; Friend et al., 1996; Labat-Moleur et al., 1996; Matsui et al., 1997; Zuhorn et al., 2002). The uptake mechanisms are, in general, closely linked with the intracellular trafficking and the fate of the vectors. A promising strategy for increasing the efficiency of nonviral vectors is to target certain uptake pathways that improve the intracellular fate of the particles. Such a strategy requires a comprehensive understanding of the different uptake pathways and the subsequent intracellular events in each case.

In this review, we attempt to relate the biological aspects of the uptake of macromolecules to the pharmaceutical aspects of molecular design in gene delivery. Different uptake pathways used for the internalization of different nonviral gene vectors and the intracellular trafficking are described in each case. These descriptions will be followed by a summary of the available knowledge of the uptake mechanism and intracellular trafficking of common classes of nonviral vectors, focusing mainly on the use of cationic lipids and polymers and comparing the different steps leading to transfection in each case.

**II. Uptake Pathways for Nonviral Gene Delivery**

Most nonviral gene vectors cannot readily cross the plasma membrane due to their large size and hydrophilic nature. Endocytosis (the vesicular uptake of extracellular macromolecules) has been established as the main mechanism for the internalization of nonviral vectors into the cells (Friend et al., 1996; Labat-Moleur et al., 1996; Zuhorn et al., 2002). Multiple mechanisms for endocytosis have been described to date (Fig. 1) (Lamaze and Schmid, 1995; Conner and Schmid, 2003). After endocytosis, the internalized molecules tend to be trapped in intracellular vesicles and eventually fuse with lysosomes where they are degraded (Bally et al., 1999). Therefore, the problem of gene delivery involves not only the cellular uptake of genes but also their intracellular availability at the target sites. Special devices, such as liposomes or peptides, that can enhance the cytosolic release of internalized molecules are

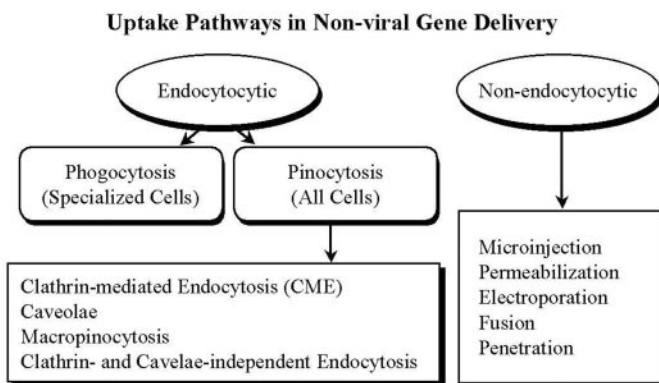


FIG. 1. Different uptake pathways in nonviral gene delivery.

essential for achieving successful gene delivery after endocytosis-mediated uptake (Smith et al., 1993a; Plank et al., 1994). To avoid the degradation problem associated with endocytosis, other nonendocytic strategies can be used to deliver genes in a manner that circumvents endocytosis (Fig. 1) (Dokka and Rojanasakul, 2000). In the following section, the different endocytic as well as the nonendocytic uptake pathways used in gene delivery are discussed.

### A. Endocytic Uptake Pathways

Endocytosis refers to the cellular uptake of macromolecules and solutes into membrane-bound vesicles derived by the invagination and pinching off of pieces of the plasma membrane. Kinetically, three modes of endocytosis can be defined: fluid-phase, adsorptive, and receptor-mediated endocytosis (Amyere et al., 2002). Fluid-phase endocytosis refers to the bulk uptake of solutes in the exact proportion to their concentration in the extracellular fluid. This is a low-efficiency and nonspecific process. In contrast, in adsorptive and receptor-mediated endocytosis, macromolecules are bound to the cell surface and concentrated before internalization. In adsorptive endocytosis, molecules preferentially interact with generic complementary binding sites (e.g., by lectin or charged interaction). The bound molecules then largely follow the fate of plasma membrane. In receptor-mediated endocytosis, certain ligands can bind to receptors on the cell surface and become concentrated before internalization. The efficiency of receptor-mediated endocytosis reflects both the affinity of the ligand-receptor interaction and the concentration of these complexes in clathrin-coated pits.

Endocytosis can also be classified into two broad categories, phagocytosis or cell eating (the uptake of large particles) and pinocytosis or cell drinking (the uptake of fluid and solutes) (Fig. 1) (Conner and Schmid, 2003). Phagocytosis is typically restricted to specialized mammalian cells, whereas pinocytosis occurs in all cells (Conner and Schmid, 2003). Therefore, the terms *endocytosis* and *pinocytosis* are occasionally considered to be synonymous (Lamaze and Schmid, 1995). At least four morphologically distinct pinocytic pathways have been characterized: clathrin-mediated endocytosis, caveolae, macropinocytosis, and clathrin/caveolae-independent endocytosis (Lamaze and Schmid, 1995). They differ in the composition of the coat (if any), in the size of the detached vesicles, and in the fate of the internalized particles. Different endocytic pathways used in gene delivery are summarized below.

1. *Clathrin-Mediated Endocytosis*. Clathrin-mediated endocytosis (CME<sup>1</sup>) is the major and best-charac-

terized endocytic pathway (Lamaze and Schmid, 1995; Takei and Haucke, 2001). CME occurs constitutively in all mammalian cells and carries out the continuous uptake of essential nutrients, antigens, growth factors, and pathogens (Takei and Haucke, 2001). The most common examples of molecules that are internalized by CME are the cholesterol-laden low-density lipoprotein (LDL) that binds to LDL receptors, and the iron-laden transferrin (Tf) that binds to Tf receptors (Schmid, 1997; Brodsky et al., 2001). CME was previously referred to as *receptor-mediated endocytosis*, but it is now clear that this is a misnomer, because most pinocytic pathways involve receptor-ligand interactions (Parton et al., 1994; Subtil et al., 1994).

Generally, the first step of internalization through CME is the strong binding of a ligand to a specific cell surface receptor. This results in the clustering of the ligand-receptor complexes in coated pits on the plasma membrane, which are formed by the assembly of cytosolic coat proteins, the main assembly units being clathrin, which form a polygonal lattice in the surface of the membrane, and adaptor protein complexes, which mediate the assembly of the clathrin-lattice on the membrane (Takei and Haucke, 2001; Conner and Schmid, 2003). The coated pits then invaginate and pinch off from the plasma membrane to form intracellular clathrin-coated vesicles (CCVs) (Fig. 2). CCVs carry concentrated receptor-ligand complexes into the cells. They range in size from ~100 to 150 nm in diameter and are characterized

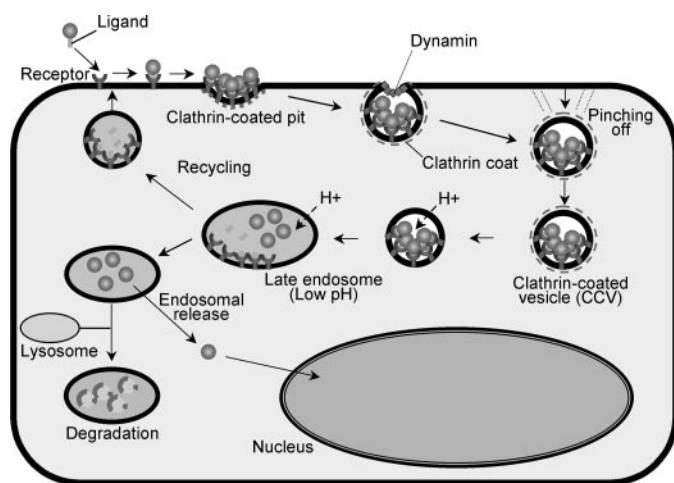


FIG. 2. Receptor (clathrin)-mediated endocytosis. The ligand first strongly binds to a specific cell surface receptor. This results in the clustering of the ligand-receptor complexes in coated pits on the plasma membrane. The coated pits then invaginate and pinch off of the plasma membrane, aided by dynamin, to form intracellular CCVs. The clathrin coat then depolymerizes, resulting in an early endosomes. Molecules entering by this pathway experience a drop in pH from neutral to pH 5.9 to 6 in the lumen of early endosomes, with a further reduction to pH 5 during the progression from late endosomes to lysosomes. The low pH in endosomes causes the ligands to dissociate from the receptors. A receptor-rich region buds off to form a separate vesicle that recycles the receptors back to the cell membrane. The vesicles then fuse with other late endosomes and eventually fuse with lysosomes in which the particles are degraded. For receptor-mediated gene delivery, a device that can facilitate the release of the gene to the cytosol is essential; otherwise, the gene cannot reach the target site such as the nucleus.

<sup>1</sup> Abbreviations: CME, clathrin-mediated endocytosis; LDL, low-density lipoprotein; Tf, transferrin; CCV, clathrin-coated vesicle; PEI, polyethyleneimine; SV40, simian virus 40; PTD, protein transduction domain; His-pLK, histidylated poly-L-lysine; CIDIQ, confocal image-assisted three-dimensionally integrated quantification; STR-R8, stearylated-octaarginine; R8, octaarginine; DOPE, dioleoylphosphatidylethanolamine; PLL, poly-L-lysine.

by the presence of a polygonal clathrin coat (Takei and Haucke, 2001). The clathrin coat then depolymerizes, resulting in early endosomes, which fuse with each other or with other preexisting endosomes to form late endosomes that further fuse with lysosomes. Molecules entering via this pathway will rapidly experience a drop in pH from neutral to pH 5.9 to 6.0 in the lumen of early endosomes, with a further reduction to pH 5 during progression from late endosomes to lysosomes (Maxfield and McGraw, 2004). Within the endosomes, ligands and receptors are sorted to their appropriate cellular destinations, such as lysosomes, the Golgi apparatus, the nucleus, or the cell surface membrane. For example, early endosomes containing LDL fuse with late endosomes, where the low pH causes the LDL particles to dissociate from the LDL receptors. A receptor-rich region buds off to form a separate vesicle that recycles the receptors back to the cell membrane (Goldstein et al., 1985). The vesicles containing LDL particles fuse with other late endosomes and eventually fuse with lysosomes in which the particles are degraded, ultimately producing cholesterol (Goldstein et al., 1985).

CME is a highly regulated process. Similar to other endocytic routes, CME is an energy-dependent process, and the assembly of the clathrin lattice on the membrane is essential for the detachment of the vesicles (Takei and Haucke, 2001). Reagents that dissociate clathrin and adaptor protein complexes from the membrane specifically inhibit the CME (Lamaze and Schmid, 1995). The GTPase dynamin is required for CME because it collaborates with coat constituents in mediating vesicle budding (Damke et al., 1994). It has been suggested that the self-assembly of dynamin functions to constrict the neck of invaginated coated pits (Takei et al., 1995). Vesicular trafficking after CME is controlled by the action of small GTPases, the Rab proteins (Zerial and McBride, 2001). Although the actin cytoskeleton is essential for CME in yeast (Ayscough, 2000), treatment of mammalian cells with actin-disrupting agents has only a partial or no effect on CCV formation (Fujimoto et al., 2000). Preventing the acidification of the endosome inhibits their fusion and maturation (Johnson et al., 1993).

In terms of gene delivery, CME can be targeted by using certain ligands, such as transferrin, which can specifically recognize certain receptors on the cell surface (Stoorvogel et al., 1991). This results in an increase in the internalization of the particles and offers the possibility of targeting specific cells that substantially overexpress the receptors. However, genes that are internalized through CME are usually trapped in endosomes followed by enzymatic degradation in lysosomes, and the final result is that genes have little or almost no access to their target sites. Actually, entrapment and degradation can be regarded as two separate barriers, because preventing lysosomal degradation results in an accumulation of genes in intra-

cellular vesicles without enhancing cytosolic release. Therefore, to reach the nucleus, genes must avoid degradation in lysosomes and must also be released from intracellular vesicles into the cytosol. Several strategies have been developed to enhance the cytosolic release of endocytosed genes (Plank et al., 1994; Tachibana et al., 1998; Simoes et al., 1999). This involves the incorporation of vesicular destructive elements to the DNA-carrier complexes, which perturb the integrity of the vesicular membrane and allow the cytosolic release of their contents, while not damaging the DNA. Some cationic polymers, e.g., polyethyleneimine (PEI), and some lipids also have the ability to enhance the cytosolic release of genes through different mechanisms as will be subsequently discussed.

**2. Caveolae-Mediated Endocytosis.** Caveolae are small, hydrophobic membrane microdomains that are rich in cholesterol and glycosphingolipids (Matveev et al., 2001; Harris et al., 2002). Classically, caveolae were defined as flask-shaped invaginations of the plasma membrane, but they can also be flat, tubular, or detached vesicles (Smart et al., 1999; Pelkmans et al., 2001; Harris et al., 2002). Caveolae are present in many cell types and are especially abundant in endothelial cells (Conner and Schmid, 2003). They are involved in several cellular processes, including cholesterol homeostasis and glycosphingolipid transport (Harris et al., 2002). Caveolae are also involved in transcytosis and endocytosis of certain viruses such as simian virus 40 (SV40), as well as some bacteria and bacterial toxins, e.g., cholera toxin. Caveolae are characterized by their association with a family of cholesterol-binding proteins called caveolins, which function to create and/or mediate these structures (Lamaze and Schmid, 1995; Matveev et al., 2001; Harris et al., 2002).

The mechanisms of caveolar internalization have been elucidated by visualizing the trafficking of the SV40 that uses caveolae to gain entry into the cells (Pelkmans et al., 2001). SV40 initially associates with the cell membrane and then becomes trapped in relatively stationary caveolae. The subsequent uptake of the virus leads to its delivery to intracellular organelles that are distinct from the classic Tf-labeled endosomes (Fig. 3). The presence of caveolin in these organelles gave rise to the name *caveosome*. SV40 then segregates from caveolin and is sorted out of caveosomes for delivery to the endoplasmic reticulum. In general, caveolae (~50–60 nm) are highly stable and are only slowly internalized, in contrast to the rapid and dynamic nature of Tf-labeled endosomes (Conner and Schmid, 2003). Another major difference is that the caveolar uptake is a nonacidic and nondigestive route of internalization (Ferrari et al., 2003). Caveolae do not suffer a drop in pH, and most pathogens that are internalized by caveolae can be directly transported to the Golgi and/or endoplasmic reticulum, thus avoiding normal lysosomal degradation.

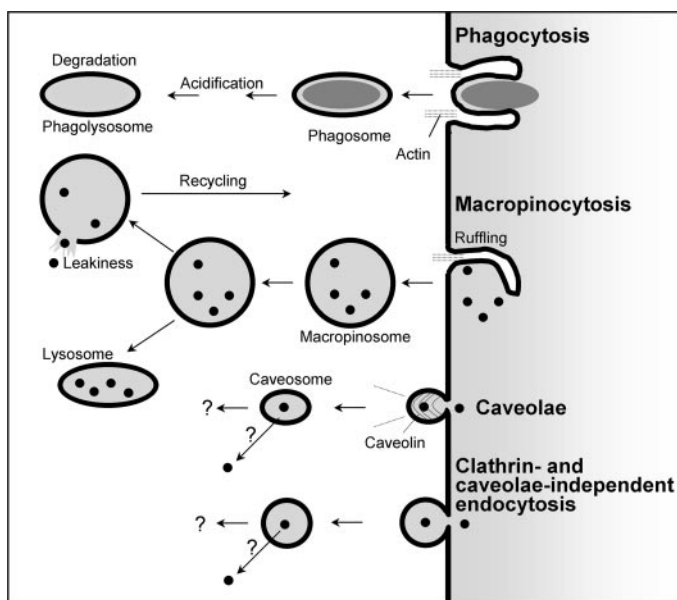


FIG. 3. Phagocytosis and clathrin-independent endocytosis. Phagocytosis is conducted primarily by specialized cells to clear large-sized pathogens or debris. Actin assembly causes the formation of cell surface extensions that zipper up around the particle to engulf it. After internalization, phagosomes mature by fusion with the components of the endocytic pathway, resulting in the formation of mature phagolysosomes where internalized particles are degraded. Three different types of clathrin-independent endocytosis have been characterized to date. Macropinocytosis refers to the formation of large and heterogeneous macropinosomes via actin-mediated cell surface ruffling. The intracellular fate of macropinosomes differs with the specific cell type. In macrophages, they merge into lysosomes. In human A431 cells, they eventually recycle most of the contents back to the cell surface. Macropinosomes are inherently leaky vesicles compared with other types of endosomes. Caveolae are typically flask-shaped invaginations of the plasma membrane characterized by the presence of caveolin. The particles initially associated with the cell membrane then became trapped in caveolae and subsequently are taken up into caveosomes. Caveolar uptake is nonacidic and nondigestive. The third type involves the formation of small vesicles that are independent of clathrin or caveolae.

The term *potocytosis* is usually associated with caveolae (Anderson et al., 1992; Matveev et al., 2001). It was first described to explain the uptake of folic acid. Potocytosis describes the internalization of small molecules without the merging of an endocytic vesicle with endosomes (Anderson et al., 1992). In uptake of folic acid, it is thought that folic acid binds to folate receptors that are clustered in invaginated caveolae, but the caveolae stay attached to the plasmalemma proper and generate a distinct microenvironment by pinching the neck region closed (Anderson et al., 1992). The ligand is then released from the receptors, and 5-methyltetrahydrofolic acid moves across the caveolar membrane where it stays in the cytosol after modification with polyglutamate, and the caveolae begin to reopen again to the extracellular space to repeat the cycle. Another term associated with caveolae is *lipid rafts*. Markers for lipid rafts are frequently found within caveolae. In general, caveolin-containing rafts are referred to as caveolae, whereas caveolin-devoid rafts are denoted by a variety of names such as glycolipid-enriched membranes and caveolae-like domains (Matveev et al., 2001).

Cholesterol is required for caveolar uptake and drugs that specifically bind to cholesterol perturb internalization through the caveolae (Schnitzer et al., 1994; Lamaze and Schmid, 1995). Caveolae also depend on the actin cytoskeleton, and drugs that cause the depolymerization of the actin cytoskeleton such as cytochalasin D can inhibit caveolae uptake without affecting clathrin-mediated endocytosis (Parton et al., 1994). Genestein, a tyrosine kinase inhibitor, can also inhibit caveolae (Orlandi and Fishman, 1998).

It is generally believed that caveolar uptake does not lead to lysosomal degradation (Harris et al., 2002; Ferrari et al., 2003). Therefore, this pathway seems to be advantageous in terms of DNA delivery. Evidence supporting the existence of a role of caveolae in the uptake of cationic polymer-DNA complexes and the class of protein transduction domains (PTDs), such as the TAT peptide, have appeared (Ferrari et al., 2003; Fittipaldi et al., 2003; Rejman et al., 2005). Another report suggested that large particles (500 nm) are preferentially taken up through caveolae where they do not suffer lysosomal degradation (Rejman et al., 2004). However, caveolae are slowly internalized and small in size, and their fluid-phase volume is small. Thus, it is unlikely that they contribute significantly to constitutive endocytosis, although the situation is different in endothelial cells in which caveolae constitute 10 to 20% of the cell surface (Conner and Schmid, 2003). Caveolae-mediated endocytosis is still a promising strategy for gene delivery especially if the internalization can be increased, possibly through the use of specific receptors for caveolae.

**3. Macropinocytosis.** Macropinocytosis refers to the formation of large endocytic vesicles of irregular size and shape, generated by actin-driven invagination of the plasma membrane (Swanson and Watts, 1995; Amyere et al., 2002). Macropinocytosis usually accompanies cell surface ruffling that is induced in many cell types upon stimulation by growth factors or other signals (Swanson and Watts, 1995; Conner and Schmid, 2003). A ruffle is formed by a linear band of outward-directed actin polymerization near the plasma membrane, which lengthens into a planar extension of the cell surface. After stimulation by any mitogenic factor, the ruffles become longer and broader and frequently close into large macropinosomes (Fig. 3) (Swanson and Watts, 1995). Macropinosomes have no coat and do not concentrate receptors. They vary in size, sometimes being as large as 5  $\mu\text{m}$  in diameter. Because they are relatively large, macropinocytosis is an efficient route for the nonselective endocytosis of solute macromolecules (Conner and Schmid, 2003). Macropinocytosis fulfills diverse functions, especially when massive fluid-phase endocytosis is necessary. This route facilitates the bulk uptake of soluble antigens by immature dendritic cells (Conner and Schmid, 2003). Some pathogens trigger macropinocytosis to facilitate their own uptake.

After the formation of macropinosomes, these vesicles lose their F-actin and their intracellular fate differs, depending on the cell type (Meier and Greber, 2003; Swanson and Watts, 1995). In macrophages, they move toward the center of the cell, shrink by loss of water, become acidified, and then completely merge into the lysosomal compartment (Meier and Greber, 2003). In human A431 cells, they do not interact with endocytic compartments other than macropinosomes (Swanson and Watts, 1995). They constitute a distinct vesicle population, which eventually recycles most of its contents back to the cell surface. Although the pH of macropinosomes decreases, they do not fuse into lysosomes in this case. Macropinosomes are thought to be inherently leaky vesicles compared with other types of endosomes (Wadia et al., 2004).

Ruffling is dependent on actin cytoskeleton; therefore, drugs that disrupt the actin cytoskeleton can inhibit macropinocytosis. The ruffling response is also dependent on protein kinase C (Conner and Schmid, 2003). Macropinocytosis can be inhibited by amiloride and its analogs, which inhibit the  $\text{Na}^+/\text{H}^+$  exchange protein in the plasma membrane (Hewlett et al., 1994).

Macropinocytosis has recently received attention as an entry route for gene and drug delivery. Recent reports have demonstrated that the uptake of the TAT peptide and its cargos occurs by macropinocytosis (Kaplan et al., 2005; Nakase et al., 2004; Wadia et al., 2004). This pathway provides some advantageous aspects such as the increased uptake of macromolecules, the avoidance of lysosomal degradation and the ease of escape from macropinosomes because of their relatively leaky nature. We have obtained data showing that, when uptake through macropinocytosis is blocked, gene expression mediated by an octaarginine peptide is impaired (Khalil et al., 2006), suggesting that this route is more productive for transfection. In contrast, a recent report has shown that macropinocytosis impaired gene expression mediated by histidylated poly-L-lysine (His-pLK), a polymer that requires an acidic pH for DNA endosomal release (Goncalves et al., 2004). It is likely that the lumen of macropinosomes is not sufficiently acidic to allow DNA delivery to the cytosol in the case of His-pLK, which is probably not the case with the octaarginine peptide. The role of macropinocytosis in gene delivery and the mechanism of macropinosomal escape are currently under investigation in our laboratory.

**4. Phagocytosis.** Phagocytosis in mammalian cells is conducted primarily by specialized cells, including macrophages, monocytes, and neutrophils, that function to clear large ( $>0.5 \mu\text{m}$ ) pathogens such as bacteria or yeast or large debris such as dead cells and arterial fat deposits (Allen and Aderem, 1996). Particle internalization is initiated by the interaction of specific receptors on the phagocyte with ligands on the surface of the particle. This triggers actin assembly and the formation of cell surface extensions that zipper up around the particle to

engulf it (Fig. 3). After internalization, actin is shed from phagosomes, which then mature by a series of fusion and fission events involving the components of the endocytic pathway, resulting in the formation of mature phagolysosomes where internalized particles are degraded (Allen and Aderem, 1996). Phagosome trafficking occurs primarily in association with microtubules, and its maturation requires a coordinated interaction between actin-based and tubulin-based cytoskeletons (Conner and Schmid, 2003). Several cell surface receptors are involved in phagocytosis; the most extensively studied of which are the opsonic receptors, including Fc receptors and complement receptors (Allen and Aderem, 1996).

Because it is primarily performed by specialized cells, phagocytosis is not expected to play a significant role in gene delivery. However, a phagocytosis-like mechanism was proposed for the uptake of large cationic lipid-DNA complexes (lipoplexes) and PEI polyplexes (Matsui et al., 1997; Kopatz et al., 2004). The proposed mechanism is dependent on the actin cytoskeleton and can explain the uptake of large lipoplexes, larger than can be taken up by the classic CME.

**5. Receptor-Mediated Endocytosis.** Although most receptors are clearly internalized by clathrin-coated pits, other pinocytic pathways are capable of selective receptor-mediated endocytosis events (Parton et al., 1994; Subtil et al., 1994). For example, the internalization of interleukin-2 into lymphocytes was partially inhibited by treatments that disrupt clathrin-mediated endocytosis, suggesting that a clathrin-independent mechanism significantly contributes to the efficient internalization of interleukin-2 receptors (Subtil et al., 1994). The targeting of some glycosphosphatidylinositol-anchored proteins and receptors to caveolae might also be induced by a ligand, such as the GM1-binding subunit of cholera toxin (Parton et al., 1994).

The use of receptor-mediated endocytosis is a promising approach for the introduction of DNA into defined cell populations. For example, hepatocytes exclusively express large numbers of high-affinity cell surface receptors that bind to and subsequently internalize asialoglycoproteins (Perales et al., 1997). Introduction of a galactose moiety into a gene delivery system can produce liver-parenchymal cell-specific gene transfection. Mannose receptor-mediated gene transfection is another approach for targeting macrophages, which overexpress mannose receptors on their surface (Kawakami et al., 2004). Tf, an iron-binding glycoprotein, has been used as a tumor-targeting ligand for gene delivery systems (Wagner et al., 1992; Kakudo et al., 2004). Tf receptors are overexpressed in rapidly dividing cells due to the increased cellular need for iron. The folate receptor is another example of receptors overexpressed in tumor cells, and it can be used for tumor targeting (Lee and Huang, 1996; Cho et al., 2005). Although receptor-mediated endocytosis is a promising approach for drug tar-

getting, most of the currently used ligands are internalized by clathrin-mediated endocytosis, and the poor intracellular trafficking associated with this significantly limits the transfection activities of the systems. Therefore, functional devices that increase the cytosolic delivery of genes are needed. For example, the intracellular fate of Tf-liposomes was improved by adding GALA, a pH-sensitive fusigenic peptide, which enhances the endosomal escape in response to the low pH in endosomes (Kakudo et al., 2004; Li et al., 2004). Exploring and targeting new receptors that can be internalized by clathrin-independent endocytosis are likely to provide more efficient systems because these uptake mechanisms are relatively unaffected by lysosomal degradation.

### B. Nonendocytic Delivery

Although the endocytosis-mediated uptake of macromolecules is an efficient cellular uptake pathway, internalized molecules suffer from poor availability at the target sites such as the nucleus. Therefore, it would be more advantageous to bypass the endocytic pathway and somehow achieve the cytosolic delivery of genes using other pathways. Microinjection is a technique that permits the rapid delivery of genes to the cytosol or the nucleus (Kleuss et al., 1991; Leonetti et al., 1991). Each cell is injected with the gene using glass capillary pipettes. Permeabilization is another technique of nonendocytic delivery. In this technique, pore-forming agents, such as streptolysin O or anionic peptides similar to the N-terminal segment of the HA2 subunit of the influenza virus hemagglutinin, which have the ability to fuse with the plasma membrane, are used to induce transmembrane channels or large apertures in the cell membrane, which then allow the entry of large molecules (Barry et al., 1993; Midoux et al., 1995). A third technique is the use of electroporation, which involves the use of an electric field to open pores in the cell (Bergan et al., 1993). However, these three techniques are highly invasive and cannot be used for *in vivo* gene delivery. The use of noninvasive nonendocytic cellular uptake is anticipated in the area of gene delivery.

Earlier work has suggested that the lipoplex-mediated delivery of genes occurs through the fusion of the lipids with the cell membrane and the direct release of DNA to the cytosol before entering the endocytic pathway (Felgner et al., 1987, 1995). However, more recent reports clearly show a significant involvement of endocytosis in the uptake, suggesting that fusion with the cell membrane contributes minimally to the overall internalization of genes (Friend et al., 1996; Labat-Moleur et al., 1996; Zuhorn et al., 2002). A class of cationic peptides, the PTDs, such as the TAT, penetratin, and VP22 peptides, may have the ability to be taken up by cells without endocytosis events (Vives et al., 1997; Thoren et al., 2003; Nakase et al., 2004; Brooks et al., 2005; Gupta et al., 2005). It was initially suggested that

these peptides directly penetrate cell membranes by an energy-independent route (Vives et al., 1997). A mechanism involving the direct penetration of the lipid bilayer caused by the localized positive charge of the peptide was proposed to explain this uptake (Trehin and Merkle, 2004). An inverted micelle-driven delivery was also proposed for the uptake of the penetratin peptide (Derossi et al., 1996). However, we have previously shown that the uptake mechanism of an octaarginine peptide, a prototype of the PTDs, was dramatically changed by N-terminal stearylation and complexation with DNA (Khalil et al., 2004). This raised a serious issue concerning the ability of the peptide to retain its activity after modification, conjugation, or complexation with other molecules. Nevertheless, according to a recent reevaluation of the uptake of these peptides and their cargos, more evidence appeared to suggest that endocytosis is the major uptake pathway (Lundberg et al., 2003; Richard et al., 2003). The possibility of the energy-independent uptake of these peptides and their cargos, however, cannot be excluded (Thoren et al., 2003), and it may be possible that certain factors, which may affect the uptake mechanism, should be optimized to achieve a successful nonendocytic delivery.

## III. Tools to Study Intracellular Trafficking in Nonviral Gene Delivery

### A. Perturbation of the Endocytosis-Mediated Uptake and Intracellular Trafficking

Certain cell treatments can inhibit internalization via endocytosis, which is generally useful in determining the uptake pathways (Table 1). These treatments should be used with caution, because they usually show cell type variations and sometimes cause nonspecific toxicity. In general, endocytic uptake is an energy-dependent mechanism. Therefore, it can be strongly inhibited by lowering the temperature or by the use of metabolic inhibitors to deplete the ATP pool (Saraste et al., 1986). Some treatments can specifically inhibit a certain endocytic pathway. For example, treatments that cause the dissociation of the clathrin lattice can specifically inhibit CME (Lamaze and Schmid, 1995). These treatments include potassium depletion, hypertonicity, cytosol acidification, and the use of chlorpromazine (Larkin et al., 1983; Sandvig et al., 1987; Heuser and Anderson, 1989; Wang et al., 1993). Some reports, however, suggest that potassium depletion can also inhibit phagocytosis (Matsui et al., 1997). The overexpression of a dominant negative mutant of dynamin potently blocks CME (Damke et al., 1994). Drugs that specifically bind, sequester, or deplete cholesterol such as filipin, nystatin, and methyl- $\beta$ -cyclodextrin, respectively, perturb internalization through the caveolae (Lamaze and Schmid, 1995). However, the specificity of these drugs is not always certain. For example, nystatin was shown to inhibit macropinocytosis and methyl- $\beta$ -cyclodextrin was shown to inhibit

TABLE 1  
*Perturbation of endocytosis and intracellular trafficking*

Treatment	Effect	Mechanism	Reference
Low temperature	General inhibitor of endocytosis	Energy depletion	Lamaze and Schmid (1995)
Metabolic inhibitors	General inhibitor of endocytosis	Energy depletion	Saraste et al. (1986)
Potassium depletion	Specific inhibitor of CME	Dissociation of clathrin lattice	Larkin et al. (1983)
Cytosol acidification	Specific inhibitor of CME	Dissociation of clathrin lattice	Sandvig et al. (1987)
Hypertonic medium	Specific inhibitor of CME	Dissociation of clathrin lattice	Heuser and Anderson (1989)
Chlorpromazine	Specific inhibitor of CME	Dissociation of clathrin lattice	Wang et al. (1993)
Filipin	Specific inhibitor of caveolae	Cholesterol binding	Lamaze and Schmid (1995)
Nystatin	Inhibitor of caveolae	Sequester cholesterol	Lamaze and Schmid (1995)
Methyl- $\beta$ -cyclodextrin	Inhibitor of caveolae	Deplete cholesterol	Lamaze and Schmid (1995); Zuhorn et al., (2002)
Genestein	Inhibitor of caveolae	Tyrosine kinase inhibitor	Orlandi and Fishman (1998)
Cytochalasins	Inhibitors of caveolae and macropinocytosis	Actin depolymerization	Parton et al. (1994)
Amiloride	Specific inhibitor of macropinocytosis	Inhibits the Na <sup>+</sup> /H <sup>+</sup> exchange protein	Hewlett et al. (1994)
Phorbol esters	Specific stimulators of macropinocytosis	Protein kinase C activators	Lamaze and Schmid (1995)
Wortmannin	Inhibitor of macropinocytosis	Phosphatidyl inositol-3-phosphate inhibitor	Arcaro and Wymann (1993)
Monensin	Inhibitor of endosome maturation	Prevents endosome acidification	Mollenhauer et al. (1990)
Chloroquine	Disrupting endosomes and lysosomes	Prevents endosome acidification and causes swelling to endosomes and lysosomes	de Duve et al. (1974); Wattiaux et al. (2000)

the formation and budding of clathrin-coated pits (Zuhorn et al., 2002; Wadia et al., 2004). The effect of filipin is dose-dependent and possibly loses its specificity at higher doses (Lamaze and Schmid, 1995). Cytochalasins cause the depolymerization of the actin cytoskeleton and inhibit uptake through the caveolae and macropinocytosis without affecting clathrin-mediated endocytosis (Parton et al., 1994). Genestein, a kinase inhibitor, can also inhibit caveolae (Orlandi and Fishman, 1998), although its specificity is doubtful. Macropinocytosis can be inhibited by amiloride and its analogs, which inhibit the Na<sup>+</sup>/H<sup>+</sup> exchange protein in the plasma membrane (Hewlett et al., 1994). Activators of protein kinase C such as phorbol esters and diacylglycerol can stimulate macropinocytosis. Nocodazole causes the depolymerization of microtubules whereas paclitaxel (Taxol) confers stability (Peterson and Mitchison, 2002). Wortmannin is a phosphatidyl inositol-3-phosphate inhibitor, which can inhibit macropinocytosis (Arcaro and Wymann, 1993). Monensin, bafilomycin A, and nigracin can inhibit the acidification of endosomes, thus preventing their maturation and fusion into lysosomes (Tartakoff, 1983; Drose and Altendorf, 1997). Treatments such as ammonium chloride, methylamine, propylamine, and chloroquine accumulate in endosomes/lysosomes due to their low pH and prevent further acidification of these vesicles (Wattiaux et al., 2000). Chloroquine causes the swelling and disruption of endocytic vesicles by osmotic effects (de Duve et al., 1974).

*B. Quantitative Evaluation of Intracellular Trafficking*

To optimize a gene delivery system, it is important to collect sufficient information about its intracellular trafficking to permit the rate-limiting steps in transfection to be defined. Therefore, a quantitative evaluation of intracellular trafficking is required to successfully design a gene delivery system. Quantification of DNA in the nucleus can be performed using a combination of the polymerase chain reaction and Southern blotting (Tachi-

vana et al., 2001). However, only a few reports are available concerning the quantification of DNA in other intracellular compartments, such as endosomes and lysosomes. The subcellular fractionation of different compartments can be used for this purpose; but this technique has some disadvantages, such as the complexity of the procedures and the mutual contamination of each compartment, which limit its use in practical applications. We recently proposed a novel strategy for simultaneously quantifying the distribution of DNA in the cytosol, endosomes/lysosomes, and nucleus, using sequential Z-series images captured by confocal laser scanning microscopy [confocal image-assisted three-dimensionally integrated quantification (CIDIQ)]. Details of this methodology are described elsewhere (Akita et al., 2004). A rhodamine-labeled DNA is typically used in the transfection, and different intracellular compartments are labeled with different fluorescent markers. For example, the acidic compartments (endosomes/lysosomes) can be stained with pH-sensitive probes, such as Lyso-Sensor (green), and the nucleus can be stained with a third marker, such as Hoechst 33258 (blue). DNA particles that are colocalized with the endosomes/lysosomes appear yellow, and those colocalized with the nucleus appear pink, whereas DNA in the cytosol appears red. Cells are scanned three-dimensionally, and images of different sections are recorded. For the quantification of DNA in each compartment, the pixel areas of the clusters are used as an index for the amount of DNA. The total pixel area for the clusters of plasmid DNA in each subcellular compartment is first determined in each xy-plane. These values are then further integrated to give the amount of DNA in each organelle, in a single cell.

This novel methodology was successfully used to relate the intracellular trafficking of three gene vectors to the expression levels of the transgene. The gene expression level obtained after the complexation of DNA with a stearylated-octaarginine (STR-R8) was considerably higher than that for DNA complexed with unmodified

octaarginine (R8), whereas it was much less than the DNA complexed with the commercially available LipofectAMINE PLUS reagent. To determine which process is the rate-determining one, CIDIQ was applied to analyze the intracellular trafficking of DNA, transfected by R8, STR-R8, and LipofectAMINE PLUS. Most of the DNA was trapped by endosomes/lysosomes in the case of R8. STR-R8 underwent endosomal escape followed by nuclear translocation in a time dependent manner. These data suggest that a stearyl moiety enhances the endosomal escape process. Furthermore, LipofectAMINE PLUS was the most effective for rapidly delivering DNA to the nucleus as well as the cytosol. Surprisingly, nuclear localization was observed within 1 h, which is as rapid as that for an adenovirus. This phenomenon is consistent with the fact that transgene expression was observed within 3 h. Collectively; the differences in transgene expression can be readily explained by intracellular trafficking assessed by CIDIQ. Such quantitative data can also be used to assess the contribution of various pathways to overall cellular uptake, which is essential for establishing intracellular pharmacokinetic models in the future.

#### IV. Uptake Mechanisms and Intracellular Trafficking of Gene Delivery Mediated by Cationic Lipids and Polymers

After summarizing the different pathways used for the cellular uptake of macromolecules, we now consider the available knowledge concerning how nonviral vectors are internalized and processed by cells to achieve transgene expression. In this section we focus mainly on the two most common methods for nonviral gene delivery: cationic lipids and cationic polymers. Both form complexes upon mixing with DNA. Cationic lipid-DNA complexes are denoted as *lipoplexes* whereas cationic polymer-DNA complexes are denoted as *polyplexes*. The use of cationic lipids in gene delivery was first introduced by Felgner et al. in 1987. Cationic polymers were introduced in the same year by Wu and Wu (1987) and were further expanded by a second generation, PEI, by Behr and coworkers in 1995 (Boussif et al., 1995). New methods have been further developed in which the cationic lipid and polymers are combined in one system (Lee and Huang, 1996; Kamiya et al., 2003; Kogure et al., 2004). An ideal nonviral vector was proposed based on both systems in combination with other functional devices to overcome extracellular and intracellular barriers (Kamiya et al., 2003).

##### A. Interaction between DNA and Cationic Lipids or Polymers

Cationic lipids are typically used in the form of cationic liposomes. All cationic lipids possess hydrophobic groups, which may be either one or two fatty acid or alkyl moieties that are 12 to 18 carbons in length, in

addition to a positively charged polar head group. The hydrophobic moieties and head groups cause the cationic lipids to assemble into bilayer vesicles (liposomes) when are dispersed in aqueous solutions. However, many cationic liposomes cannot form liposomes alone and are normally accompanied by a neutral lipid such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol. DOPE is frequently useful because it can fuse with other lipids when exposed to a low pH, such as in endosomes, which aid in the release of the associated DNA into the cytosol (Cullis et al., 1986; Farhood et al., 1995). Cholesterol provides structural stability, and there is evidence that it can influence targeting in vivo via scavenger receptors (Allen and Chonn, 1987; Hug and Sleight, 1991).

Lipoplexes are typically formed by direct mixing between cationic liposomes and DNA solutions. Positively charged liposomes bind to negatively charged phosphate molecules on the DNA backbone through electrostatic interactions. Generally, complexes are formed with a slight excess positive charge to permit them to interact with the negatively charged cell surface. The ratio between the cationic charge of the liposome and the negative charge of the DNA usually controls the size of lipoplexes (Almofti et al., 2003). At high positive or negative charge ratios, relatively small complexes are formed, whereas large aggregates are usually formed when the net charge is close to neutrality (Eastman et al., 1997; Almofti et al., 2003). The cationic liposomes used are typically small (~100 nm) before adding to DNA; however, complexes formed with DNA exhibit diameters that range from as small as 200 nm to structures as large as 2  $\mu\text{m}$  (Wasan et al., 1996). The formation of lipoplexes is generally difficult to control, and different structures are produced in the same lipoplex preparation. The proposed model for describing the interaction between cationic liposomes and DNA involves the following. First, liposomes cause a compaction of the DNA molecules and charge neutralization. Second, neutralization may induce aggregation, resulting in the formation of a heterogeneous group of multilamellar structures of different shapes and consisting of DNA sandwiched between lipid bilayers. Third, it is proposed that DNA affects the liposomes, inducing lipid mixing and rearrangement resulting in fusion of the multilamellar structures to form large DNA-lipid complexes (Radler et al., 1997).

Usually, positively charged lipoplexes lead to more efficient gene expression by virtue of ionic interactions with the negative cell surface. In addition to the compaction and neutralization of DNA, cationic liposomes provide a protective role against extra- and intracellular nucleases. This can be attributed to the compaction and covering of DNA by the lipid bilayers (Eastman et al., 1997). Unfortunately, the positive charge of lipoplexes makes them susceptible to interaction with negative constituents in the circulation after in vivo admini-

stration, which significantly limits their use in systemic administration. Although smaller-sized lipoplexes would be expected to be more efficiently internalized via endocytosis, larger lipoplexes have been reported to improve transfection activities (Liu et al., 1997). This may be due to the greater ability of the larger particles to sediment onto the cell surface. However, the *in vivo* transfection ability of larger complexes is weak, mainly because of their inability to reach the target cells because of their large size, which also renders them more susceptible to interaction with extracellular components.

Cationic polymers can also condense and neutralize DNA. They do not have hydrophobic moieties, but they can condense the DNA more efficiently resulting in smaller DNA condensed particles. Among the many cationic polymers available, the most frequently used in gene delivery are poly-L-lysine (PLL)- and PEI-based polymers (Wu and Wu, 1987; Boussif et al., 1995). Unlike lipoplexes, polyplexes formed with PLL usually use ligands to facilitate their cellular uptake, and endosomal reagents are usually used to facilitate endosomal escape. PEI-based polyplexes are more efficient and do not require agents for endosomal escape (Klemm et al., 1998). Upon mixing with DNA, electrostatic interactions occur between the cationic charge of the polymer and the negative charge of the DNA, resulting in the formation of particles, as small as 20 to 40 nm in some cases. The size and the charge of the polyplexes depend to a greater extent on the ratio between the polymer and DNA than on the properties of the polymer (Choosakoonkriang et al., 2003). The term *lipopolyplex* has been introduced more recently and involves the condensation of DNA using a polycation followed by entrapping the polyplexes within anionic, neutral or even cationic peptide-modified liposomes (Lee and Huang, 1996; Kogure et al., 2004). This method is less toxic, probably due to the exclusion of the cationic lipids, and in some cases more efficient compared with lipoplexes or polyplexes (Ibanez et al., 1996). It combines the high compaction of polyplexes and the facilitated endosomal escape of the lipoplexes. In addition, they provide more protection to DNA. Furthermore, the liposome surface can be easily modified with targeting ligands or polyethyleneglycol to escape the recognition by opsonins as well as macrophages after systemic administration (Kiwada et al., 1998).

### B. Cellular Binding

Unless a specific targeting ligand is incorporated in the system, the binding of lipoplexes and polyplexes to the cell surface is the result of a nonspecific ionic interaction between the positive charge of the complexes and the negative charge of the cell surface. Negatively charged cell surface constituents, such as heparan sulfate proteoglycans and integrins play a role in the cellular binding of positively charged lipoplexes, poly-

plexes, or even cationic peptides, such as TAT (Behr et al., 1989; Labat-Moleur et al., 1996; Tyagi et al., 2001; Richard et al., 2005). For example, in proteoglycan-deficient mutant cells, the cellular binding of lipoplexes and polyplexes is reduced (Mislick and Baldeschwieler, 1996). The presence of soluble heparin and heparan sulfate in the medium competitively inhibits the binding. At this point, the heparan sulfate proteoglycans may act as nonspecific receptors for cationic macromolecules, but their exact role in mediating cellular uptake is not clear. There is some evidence to show that the transmembrane proteins, syndecans, may cluster to form focal points at the plasma membrane during binding to cationic particles and this clustering induces their interaction with the actin cytoskeleton, probably resulting in the formation of tension fibers. This tension provides the energy required to engulf the particles (Woods and Couchman, 1994). Similarly, a recent report suggested that the uptake of PEI polyplexes occurs through actin-mediated phagocytosis as a result of the adhesion of polyplexes to syndecan molecules followed by their clustering in lipid rafts (Kopatz et al., 2004).

### C. Cellular Uptake

The internalization mechanism of lipoplexes is not well understood. Early reports suggested that fusion between the lipids and the plasma membrane is responsible for delivering DNA directly to the cytosol (Felgner et al., 1987, 1995). It was suggested that the interaction between the liposomes and DNA or the cell membrane destabilize the liposomes, thus facilitating their fusion with each other and with other membranes. However, most of the following experimental evidence supports the involvement of endocytosis as a main entrance route (Fig. 4). For example, the use of endocytosis inhibitors significantly reduces gene expression. Furthermore, interference of the endocytic pathway with lysosomotropic reagents such as chloroquine was found to enhance the gene expression (Cotten et al., 1990). The strongest evidence comes from electron microscopy imaging of gold-labeled DNA, which clearly shows the presence of DNA in intracellular vesicles, a typical entry via endocytosis (Friend et al., 1996). In general, it is currently believed that membrane fusion is important for transfection but that most of the uptake occurs through endocytosis. Membrane fusion occurs as a result of endosome acidification and is responsible for releasing the endosome contents to the cytosol. In contrast to this general belief, Almofti et al. (2003) proposed that the uptake of lipoplexes occurs by endocytosis but that membrane fusion occurs mostly (72%) at the plasma membrane level, and it is essential for endocytosis to occur.

The current question is which pathway of endocytosis is responsible for uptake. The available data show diverse results. Rejman et al. (2005) reported that the uptake of lipoplexes formed between the cationic lipid DOTAP and DNA is inhibited by chlorpromazine and

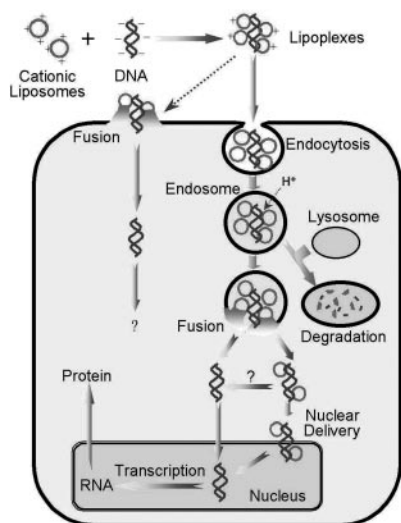


FIG. 4. Uptake and intracellular trafficking of cationic liposome/DNA complexes (lipoplexes). Lipoplexes are mainly taken up through endocytosis. The low pH of endosomes causes fusion between the liposomal and endosomal membranes. The DNA is released to the cytosol either free or associated with the lipids and is then delivered to the nucleus for translation into protein. Fusion may also occur at the plasma membrane, thus delivering the DNA directly to the cytosol.

potassium depletion but is unaffected by filipin and genestein, suggesting that the uptake occurs solely by clathrin-mediated endocytosis. Furthermore, they have shown that particles that are internalized by CME are eventually degraded in lysosomes. Earlier, Zhou and Huang (1994) suggested that the uptake of lipopoly-L-lysine lipoplexes occurs through clathrin-mediated endocytosis because the presence of the actin-depolymerizing reagent cytochalasin B increased transfection activities. In contrast to Rejman et al. (2005), Zhou and Huang (1994) suggested that CME is the most productive pathway for internalization. Zuhorn et al. (2002) have shown that lipoplex-mediated transfection occurs through cholesterol-dependent clathrin-mediated endocytosis. Cholesterol depletion with methyl- $\beta$ -cyclodextrin decreased the activities of SAINT-2-DOPE lipoplexes. This is largely indicative of nonclathrin endocytosis; however, inhibitors of caveolae such as filipin and cytochalasin D had only a slight effect on internalization. Colocalization with Tf (a CME marker) and the inhibition by potassium depletion further confirmed the involvement of CME. In contrast to these reports, Matsui et al. (1997) suggested that the uptake of LipofectACE lipoplexes occurs through phagocytosis in poorly differentiated airway epithelia cells. Similar to the uptake of large (2  $\mu$ m) microspheres, which were used as markers for phagocytosis, they found that the uptake of lipoplexes was inhibited by cytochalasin B as well as by potassium depletion.

The uptake of polyplexes also occurs through endocytosis, but without fusion with the cell membrane, and similarly shows some diverse results regarding specific uptake pathways. In general, it is believed that the uptake of PLL and PEI complexes occurs through CME.

Goncalves et al. (2004) have shown that the uptake of His-pLK polyplexes occurs through both clathrin-dependent and -independent pathways; the latter is mostly macropinocytosis, because it was inhibited by amiloride and stimulated by phorbol esters. Furthermore, they found that macropinocytosis of the polyplexes and the recycling of DNA impaired the transfection and concluded that CME is the most productive pathway. Rejman et al. (2005) also suggested that the uptake of PEI polyplexes occurs through both clathrin-dependent and -independent pathways; however, they suggested that the latter mechanism involves the caveolae because it was inhibited by filipin and genestein. Another difference arises from their finding that CME is less productive since caveolar internalization escapes lysosomes, thus leading to efficient transfection. The diversity of results suggests that a variety of factors may affect the actual mechanism. However, in general these results collectively suggest that endosomal escape is a significant barrier to lipoplex- and polyplex-mediated transfection.

#### D. Endosomal Escape

After internalization via endocytosis, the internalized molecules exist in endosomes with no access to the cytosol or the nucleus. These endosomes either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, escape from endosomes is essential for efficient transfection. Lipoplexes containing the pH-sensitive fusigenic lipid DOPE can release the associated DNA into the cytosol (Farhood et al., 1995). DOPE forms a stable lipid bilayer at physiological pH  $\sim$ 7; however, at an acidic pH 5 to 6, it undergoes a transition from a bilayer to an inverted hexagonal structure, which fuses and destabilizes the endosomal membrane, releasing its contents to the cytosol (Cullis et al., 1986). Evidence exists to show that fusion with the endosomal membrane is essential for DOPE-containing lipoplexes (Wrobel and Collins, 1995; Almofti et al., 2003). It is possible that only DNA or the lipoplex as a whole will be released to the cytosol after fusion. If lipoplexes are released, the dissociation of DNA must occur in the cytosol or even at the nuclear membrane to achieve transfection.

Similar to DOPE, which has an intrinsic ability to cause endosomal release, the polycation PEI has the same ability, although through a different mechanism (Behr et al., 1989). This is evident by the observation that transfection with PEI polyplexes is not improved by fusigenic peptides or chloroquine (Kichler et al., 2001). A proton sponge hypothesis was proposed by Behr and coworkers to explain this phenomenon (Boussif et al., 1995). This hypothesis suggests that PEI becomes more protonated at low pH as in endosomes. This protonation triggers an influx of  $\text{Cl}^-$  ions with protons leading to a water influx and finally the swelling and rupturing of the endosomes.

Lipoplexes lacking fusogenic lipids and polyplexes without proton sponge ability are not released efficiently into the cytosol unless additional functional devices for endosomal release are used. Viruses such as the influenza and adeno virus use the acidic pH of endosomes to induce endosomal disruption or fusion. Similarly, functional devices, which use the acidic pH of the endosome to induce their rupture, are incorporated in these systems. An example of such functional devices is the pH-sensitive fusogenic peptides. These peptides are derived from viruses, such as the peptide derived from the N-terminal sequence of the influenza virus hemagglutinin subunit HA-2, or prepared synthetically, such as GALA or KALA (Wagner et al., 1992; Simoes et al., 1999). These peptides undergo conformational changes at the low pH in the endosomes to interact with and perturb the endosomal membrane. The incorporation of the influenza HA-2 subunit augmented the Tf-PLL-mediated gene transfer. The presence of the cholesterol-GALA peptide on the liposomal membrane effectively enhanced the endosomal release of the liposome contents (Kakudo et al., 2004). Another approach for increasing endosomal escape is the use of lysosomotropic reagents such as chloroquine. It is a weak, hydrophobic base, which enters the lysosomes and becomes protonated in its acidic environment. This triggers a swelling of lysosomes and destabilization of their membranes. Chloroquine also inhibits the acidification and maturation of endosomes (Mellman et al., 1986), thus retarding the lysosomal degradation of genes. The addition of chloroquine to the transfection medium usually favors transfection. This may be due to the destabilization of the endosomal/lysosomal membranes and/or to a slowdown in the translocation of DNA to the lysosomes. However, depending on the system used, chloroquine may decrease the transfection, such as with PEI polyplexes, by inhibiting endosome acidification, which is required for the release of DNA from endosomes. It is worth mentioning, that the use of chloroquine and similar lysosomotropic reagents is usually associated with toxicity, which limits their use in actual applications.

### *E. Nuclear Delivery*

The nuclear envelope contains nuclear pores with a passive transport limit of 70 kDa molecular mass or ~10 nm diameter (Melchior and Gerace, 1995). This is much smaller than the size of DNA, even when condensed in lipoplexes or polyplexes. Microinjection of plasmid DNA encoding  $\beta$ -galactosidase into the nucleus produced a much higher gene expression than when the same plasmid was microinjected into the cytosol (Pollard et al., 1998). This suggests that the nuclear envelope is a significant barrier against transfection. How then is the DNA delivered to the nucleus? The most widely accepted model is that cell division is an important factor in the nuclear translocation of transgenes. During mitosis, the integrity of the nuclear membrane is transiently

lost, which allows the nuclear entry of transgenes. This is the case in the in vitro transfection with dividing cells, whereas in vivo transfection usually targets differentiated nondividing cells. Therefore, the nuclear envelope cannot be neglected in in vivo situations. DNA could be detected in the nucleus in time intervals as low as 1 h after lipoplex-mediated transfection, which suggests that a different mechanism is involved in this early nuclear delivery (Akita et al., 2004). We have previously shown that lipoplexes can fuse with the nuclear membrane, thus releasing DNA to the nucleus (Kamiya et al., 2002). Targeting efforts to enhance this mechanism are needed, especially for the transfection of nondividing cells.

The nuclear injection of lipoplexes results in poor gene expression compared with injection of naked DNA (Zabner et al., 1995). This finding suggests that decondensation in the nucleus is a poor process. The generally accepted model was proposed by Xu and Szoka (1996) and involves the release of DNA from lipoplexes during endosomal release, thus delivering only naked DNA to the cytosol. In contrast to cationic lipids, the microinjection of PEI polyplexes did not affect the transgene expression when the complexes are injected into the nucleus (Zabner et al., 1995), suggesting that a rapid release of DNA from the polyplexes occurs in the nucleus, probably via an exchange with cellular DNA.

Nuclear proteins require a nuclear localization signal (NLS), which contains basic amino acids and can be recognized by cytosolic factors to mediate active transport through the nuclear pore complex (Jans and Hubner, 1996). During this active transport, the diameter of the nuclear pore complex is expanded to ~30 nm, and this allows the delivery of nuclear proteins to the nucleus (Dworetzky et al., 1988). The same approach can be used to enhance gene delivery to the nucleus. The nuclear delivery of DNA was increased by the coupling of 100 NLS peptides/kilobase pair of DNA (Sebestyen et al., 1998). The amount of the NLS peptides seems to be important in delivering the gene. In addition, the peptide should be coupled to the DNA in the case of polyplexes, because coupling of the NLS to the polycation PLL did not enhance gene expression.

## V. Conclusions

Different uptake pathways are involved in the cellular delivery of macromolecules. The contribution of certain pathways in the uptake of lipid- and polymer-mediated gene delivery is not well understood and seems to be affected by the nature and characteristics of the gene vectors. An understanding of the mechanism of uptake and intracellular trafficking is basically required for designing successful gene delivery. Receptor-mediated endocytosis is an efficient way to target a specific cell population; however, components to overcome the endocytic barriers are essential for efficient gene delivery.

Exploring new ligands to target endocytic pathways other than and superior to CME is the next step in terms of increasing the efficiency of nonviral gene delivery systems.

**Acknowledgments.** We thank Dr. M. S. Feather for helpful advice in writing the English manuscript.

## REFERENCES

- Akita H, Ito R, Khalil IA, Futaki S, and Harashima H (2004) Quantitative three-dimensional analysis of the intracellular trafficking of plasmid DNA transfected by a nonviral gene delivery system using confocal laser scanning microscopy. *Mol Ther* **9**:443–451.
- Allen LA and Aderem A (1996) Mechanisms of phagocytosis. *Curr Opin Immunol* **8**:36–40.
- Allen TM and Chonn A (1987) Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett* **223**:42–46.
- Almofiti MR, Harashima H, Shinohara Y, Almofiti A, Baba Y, and Kiwada H (2003) Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization. *Arch Biochem Biophys* **410**:246–253.
- Ameyer M, Mettlen M, Van Der Smissen P, Platek A, Payrastré B, Veithen A, and Courtoy PJ (2002) Origin, originality, functions, subversions and molecular signalling of macropinocytosis. *Int J Med Microbiol* **291**:487–494.
- Anderson RG, Kamen BA, Rothberg KG, and Lacey SW (1992) Potocytosis: sequestration and transport of small molecules by caveolae. *Science (Wash DC)* **255**:410–411.
- Arcaro A and Wymann MP (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296** (Pt 2):297–301.
- Ayscough KR (2000) Endocytosis and the development of cell polarity in yeast require a dynamic F-actin cytoskeleton. *Curr Biol* **10**:1587–1590.
- Bally MB, Harvie P, Wong FM, Kong S, Wasan EK, and Reimer DL (1999) Biological barriers to cellular delivery of lipid-based DNA carriers. *Adv Drug Deliv Rev* **38**:291–315.
- Barry EL, Gesek FA, and Friedman PA (1993) Introduction of antisense oligonucleotides into cells by permeabilization with streptolysin O. *Biotechniques* **15**:1016–1018, 1020.
- Behr JP, Demeneix B, Loeffler JP, and Perez-Mutul J (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci USA* **86**:6982–6986.
- Bergan R, Connell Y, Fahmy B, and Neckers L (1993) Electroporation enhances c-myc antisense oligodeoxynucleotide efficacy. *Nucleic Acids Res* **21**:3567–3573.
- Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, and Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* **92**:7297–7301.
- Brodsky FM, Chen CY, Kneuhl C, Towler MC, and Wakeham DE (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol* **17**:517–568.
- Brooks H, Lebleu B, and Vives E (2005) Tat peptide-mediated cellular delivery: back to basics. *Adv Drug Deliv Rev* **57**:559–577.
- Cho KC, Kim SH, Jeong JH, and Park TG (2005) Folate receptor-mediated gene delivery using folate-poly(ethylene glycol)-poly(L-lysine) conjugate. *Macromol Biosci* **5**:512–519.
- Choosakoonkriang S, Lobo BA, Koe GS, Koe JG, and Middaugh CR (2003) Biophysical characterization of PEI/DNA complexes. *J Pharm Sci* **92**:1710–1722.
- Conner SD and Schmid SL (2003) Regulated portals of entry into the cell. *Nature (Lond)* **422**:37–44.
- Cotten M, Langle-Rouault F, Kirlappos H, Wagner E, Mechtler K, Zenke M, Beug H, and Birmstiel ML (1990) Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. *Proc Natl Acad Sci USA* **87**:4033–4037.
- Cullis PR, Hope MJ, and Tilcock CP (1986) Lipid polymorphism and the roles of lipids in membranes. *Chem Phys Lipids* **40**:127–144.
- Damke H, Baba T, Warnock DE, and Schmid SL (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol* **127**:915–934.
- de Duve C de Barys T, Poole B, Trouet A, Tulkens P, and Van Hoof F (1974) Commentary: lysosomotropic agents. *Biochem Pharmacol* **23**:2495–2531.
- Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, and Prochiantz A (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* **271**:18188–18193.
- Dokka S and Rojanasakul Y (2000) Novel non-endocytic delivery of antisense oligonucleotides. *Adv Drug Deliv Rev* **44**:35–49.
- Drose S and Altendorf K (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J Exp Biol* **200**:1–8.
- Dworetzky SI, Lanford RE, and Feldherr CM (1988) The effects of variations in the number and sequence of targeting signals on nuclear uptake. *J Cell Biol* **107**:1279–1287.
- Eastman SJ, Siegel C, Tousignant J, Smith AE, Cheng SH, and Scheule RK (1997) Biophysical characterization of cationic lipid: DNA complexes. *Biochim Biophys Acta* **1325**:41–62.
- Farhood H, Serbina N, and Huang L (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* **1235**:289–295.
- Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, and Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* **84**:7413–7417.
- Felgner PL, Tsai YJ, Sukhu L, Wheeler CJ, Manthorpe M, Marshall J, and Cheng SH (1995) Improved cationic lipid formulations for in vivo gene therapy. *Ann NY Acad Sci* **772**:126–139.
- Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, and Beltram F (2003) Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol Ther* **8**:284–294.
- Fittipaldi A, Ferrari A, Zoppe M, Arcangeli C, Pellegrini V, Beltram F, and Giacca M (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. *J Biol Chem* **278**:34141–34149.
- Friend DS, Papahadjopoulos D, and Debs RJ (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim Biophys Acta* **1278**:41–50.
- Fujimoto LM, Roth R, Heuser JE, and Schmid SL (2000) Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic* **1**:161–171.
- Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, Harashima H, and Sugiura Y (2001) Stearoylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem* **12**:1005–1011.
- Goldstein JL, Brown MS, Anderson RG, Russell DW, and Schneider WJ (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu Rev Cell Biol* **1**:1–39.
- Goncalves C, Mennesson E, Fuchs R, Gorvel JP, Midoux P, and Pichon C (2004) Macropinocytosis of polyplexes and recycling of plasmid via the clathrin-dependent pathway impair the transfection efficiency of human hepatocarcinoma cells. *Mol Ther* **10**:373–385.
- Gupta B, Levchenko TS, and Torchilin VP (2005) Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv Drug Deliv Rev* **57**:637–651.
- Harris J, Werling D, Hope JC, Taylor G, and Howard CJ (2002) Caveolae and caveolin in immune cells: distribution and functions. *Trends Immunol* **23**:158–164.
- Heuser JE and Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108**:389–400.
- Hewlett LJ, Prescott AR, and Watts C (1994) The coated pit and macropinocytotic pathways serve distinct endosome populations. *J Cell Biol* **124**:689–703.
- Hug P and Sleight RG (1991) Liposomes for the transformation of eukaryotic cells. *Biochim Biophys Acta* **1097**:1–17.
- Ibanez M, Gariglio P, Chavez P, Santiago R, Wong C, and Baeza I (1996) Spermidine-condensed DNA and cone-shaped lipids improve delivery and expression of exogenous DNA transfer by liposomes. *Biochem Cell Biol* **74**:633–643.
- Jans DA and Hubner S (1996) Regulation of protein transport to the nucleus: central role of phosphorylation. *Physiol Rev* **76**:651–685.
- Johnson LS, Dunn KW, Pytowski B, and McGraw TE (1993) Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol Biol Cell* **4**:1251–1266.
- Kakudo T, Chaki S, Futaki S, Nakase I, Akaji K, Kawakami T, Maruyama K, Kamiya H, and Harashima H (2004) Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry* **43**:5618–5628.
- Kamiya H, Akita H, and Harashima H (2003) Pharmacokinetic and pharmacodynamic considerations in gene therapy. *Drug Discov Today* **8**:990–996.
- Kamiya H, Fujimura Y, Matsuoka I, and Harashima H (2002) Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. *Biochem Biophys Res Commun* **298**:591–597.
- Kaplan IM, Wadia JS, and Dowdy SF (2005) Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* **102**:247–253.
- Kawakami S, Hattori Y, Lu Y, Higuchi Y, Yamashita F, and Hashida M (2004) Effect of cationic charge on receptor-mediated transfection using mannoseylated cationic liposome/plasmid DNA complexes following the intravenous administration in mice. *Pharmazie* **59**:405–408.
- Khalil IA, Futaki S, Niwa M, Baba Y, Kaji N, Kamiya H, and Harashima H (2004) Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. *Gene Ther* **11**:636–644.
- Khalil IA, Kogure K, Futaki S, and Harashima H (2006) High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. *J Biol Chem*, in press.
- Kichler A, Leborgne C, Coeytaux E, and Danos O (2001) Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med* **3**:135–144.
- Kiwada H, Matsuo H, and Harashima H (1998) Identification of proteins mediating clearance of liposomes using a liver perfusion system. *Adv Drug Deliv Rev* **32**:61–79.
- Klemm AR, Young D, and Lloyd JB (1998) Effects of polyethylenimine on endocytosis and lysosome stability. *Biochem Pharmacol* **56**:41–46.
- Kleuss C, Hescheler J, Ewel C, Rosenthal W, Schultz G, and Wittig B (1991) Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature (Lond)* **353**:43–48.
- Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, and Harashima H (2004) Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. *J Control Release* **98**:317–323.
- Kopatz I, Remy JS, and Behr JP (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med* **6**:769–776.
- Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstemberger P, Oberling F, Brambilla E, and Behr JP (1996) An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther* **3**:1010–1017.
- Lamaze C and Schmid SL (1995) The emergence of clathrin-independent pinocytotic pathways. *Curr Opin Cell Biol* **7**:573–580.
- Larkin JM, Brown MS, Goldstein JL, and Anderson RG (1983) Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* **33**:273–285.
- Ledley FD (1994) Non-viral gene therapy. *Curr Opin Biotechnol* **5**:626–636.

- Lee RJ and Huang L (1996) Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J Biol Chem* **271**:8481–8487.
- Leonetti JP, Mechti N, Degols G, Gagnor C, and Lebleu B (1991) Intracellular distribution of microinjected antisense oligonucleotides. *Proc Natl Acad Sci USA* **88**:2702–2706.
- Li S and Huang L (2000) Nonviral gene therapy: promises and challenges. *Gene Ther* **7**:31–34.
- Li W, Nicol F, and Szoka FC Jr (2004) GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv Drug Deliv Rev* **56**:967–985.
- Liu Y, Mounkes LC, Liggitt HD, Brown CS, Solodin I, Heath TD, and Debs RJ (1997) Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol* **15**:167–173.
- Lundberg M, Wikstrom S, and Johansson M (2003) Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* **8**:143–150.
- Matsui H, Johnson LG, Randell SH, and Boucher RC (1997) Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J Biol Chem* **272**:1117–1126.
- Matveev S, Li X, Everson W, and Smart EJ (2001) The role of caveolae and caveolin in vesicle-dependent and vesicle-independent trafficking. *Adv Drug Deliv Rev* **49**:237–250.
- Maxfield FR and McGraw TE (2004) Endocytic recycling. *Nat Rev Mol Cell Biol* **5**:121–132.
- Meier O and Greber UF (2003) Adenovirus endocytosis. *J Gene Med* **5**:451–462.
- Melchior F and Gerace L (1995) Mechanisms of nuclear protein import. *Curr Opin Cell Biol* **7**:310–318.
- Mellman I, Fuchs R, and Helenius A (1986) Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem* **55**:663–700.
- Midoux P, Mayer R, and Monsigny M (1995) Membrane permeabilization by  $\alpha$ -helical peptides: a flow cytometry study. *Biochim Biophys Acta* **1239**:249–256.
- Mislick KA and Baldeschwieler JD (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci USA* **93**:12349–12354.
- Mollenhauer HH, Morre DJ, and Rowe LD (1990) Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim Biophys Acta* **1031**:225–246.
- Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, Huang L, Shu S, Gordon D, and Chang AE (1993) Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity and lack of toxicity in humans. *Proc Natl Acad Sci USA* **90**:11307–11311.
- Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, Takehashi M, Tanaka S, Ueda K, Simpson JC, et al. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol Ther* **10**:1011–1022.
- Orlandi PA and Fishman PH (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol* **141**:905–915.
- Parton RG, Joggerst B, and Simons K (1994) Regulated internalization of caveolae. *J Cell Biol* **127**:1199–1215.
- Pelkmans L, Kartenbeck J, and Helenius A (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* **3**:473–483.
- Perales JC, Grossmann GA, Molas M, Liu G, Ferkol T, Harpst J, Oda H, and Hanson RW (1997) Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. *J Biol Chem* **272**:7398–7407.
- Peterson JR and Mitchison TJ (2002) Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton. *Chem Biol* **9**:1275–1285.
- Plank C, Oberhauser B, Mechtler K, Koch C, and Wagner E (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J Biol Chem* **269**:12918–12924.
- Pollard H, Remy JS, Lousouarn G, Demolombe S, Behr JP, and Escande D (1998) Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* **273**:7507–7511.
- Radler JO, Koltover I, Salditt T, and Safinya CR (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science (Wash DC)* **275**:810–814.
- Rejman J, Bragonzi A, and Conese M (2005) Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther* **12**:468–474.
- Rejman J, Oberle V, Zuhorn IS, and Hoekstra D (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* **377**:159–169.
- Richard JP, Melikov K, Brooks H, Prevot P, Lebleu B, and Chernomordik LV (2005) Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* **280**:15300–15306.
- Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, and Lebleu B (2003) Cell-penetrating peptides: a reevaluation of the mechanism of cellular uptake. *J Biol Chem* **278**:585–590.
- Sandvig K, Olsnes S, Petersen OW, and van Deurs B (1987) Acidification of the cytosol inhibits endocytosis from coated pits. *J Cell Biol* **105**:679–689.
- Saraste J, Palade GE, and Farquhar MG (1986) Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc Natl Acad Sci USA* **83**:6425–6429.
- Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem* **66**:511–548.
- Schnitzer JE, Oh P, Pinney E, and Allard J (1994) Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis and capillary permeability of select macromolecules. *J Cell Biol* **127**:1217–1232.
- Sebestyen MG, Ludtke JJ, Bassik MC, Zhang G, Budker V, Lukhtanov EA, Hagstrom JE, and Wolff JA (1998) DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat Biotechnol* **16**:80–85.
- Simoes S, Slepushkin V, Pires P, Gaspar R de Lima MP, and Duzgunes N (1999) Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther* **6**:1798–1807.
- Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PE, Okamoto T, and Lisanti MP (1999) Caveolins, liquid-ordered domains and signal transduction. *Mol Cell Biol* **19**:7289–7304.
- Smith JG, Walzem RL, and German JB (1993a) Liposomes as agents of DNA transfer. *Biochim Biophys Acta* **1154**:327–340.
- Smith TA, Mehaffey MG, Kayda DB, Saunders JM, Yei S, Trapnell BC, McClelland A, and Kaleko M (1993b) Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nat Genet* **5**:397–402.
- Stoorvogel W, Strous GJ, Ciechanover A, and Schwartz AL (1991) Trafficking of the transferrin receptor. *Targeted Diagn Ther* **4**:267–304.
- Subtil A, Hemar A, and Dautry-Varsat A (1994) Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. *J Cell Sci* **107** (Pt 12):3461–3468.
- Swanson JA and Watts C (1995) Macropinocytosis. *Trends Cell Biol* **5**:424–428.
- Tachibana R, Harashima H, Shinohara Y, and Kiwada H (2001) Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors. *Adv Drug Deliv Rev* **52**:219–226.
- Tachibana R, Harashima H, Shono M, Azumano M, Niwa M, Futaki S, and Kiwada H (1998) Intracellular regulation of macromolecules using pH-sensitive liposomes and nuclear localization signal: qualitative and quantitative evaluation of intracellular trafficking. *Biochem Biophys Res Commun* **251**:538–544.
- Takei K and Haucke V (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol* **11**:385–391.
- Takei K, McPherson PS, Schmid SL, and De Camilli P (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTP- $\gamma$ S in nerve terminals. *Nature (Lond)* **374**:186–190.
- Tartakoff AM (1983) Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**:1026–1028.
- Thoren PE, Persson D, Isakson P, Gokors M, Onfelt A, and Norden B (2003) Uptake of analogs of penetratin, Tat(48–60) and oligoarginine in live cells. *Biochem Biophys Res Commun* **307**:100–107.
- Torchilin VP, Levchenko TS, Rammohan R, Volodina N, Papahadjopoulos-Sternberg B, and D'Souza GG (2003) Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proc Natl Acad Sci USA* **100**:1972–1977.
- Trehin R and Merkle HP (2004) Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur J Pharm Biopharm* **58**:209–223.
- Tyagi M, Rusnati M, Presta M, and Giacca M (2001) Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans. *J Biol Chem* **276**:3254–3261.
- Vives E, Brodin P, and Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272**:16010–16017.
- Wadia JS, Stan RV, and Dowdy SF (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10**:310–315.
- Wagner E, Plank C, Zatloukal K, Cotten M, and Birnstiel ML (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci USA* **89**:7934–7938.
- Wang LH, Rothberg KG, and Anderson RG (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol* **123**:1107–1117.
- Wasan EK, Reimer DL, and Bally MB (1996) Plasmid DNA is protected against ultrasonic cavitation-induced damage when complexed to cationic liposomes. *J Pharm Sci* **85**:427–433.
- Wattiaux R, Laurent N, Wattiaux-De Coninck S, and Jadot M (2000) Endosomes, lysosomes: their implication in gene transfer. *Adv Drug Deliv Rev* **41**:201–208.
- Woods A and Couchman JR (1994) Syndecan 4 heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. *Mol Biol Cell* **5**:183–192.
- Wrobel I and Collins D (1995) Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta* **1235**:296–304.
- Wu GY and Wu CH (1987) Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J Biol Chem* **262**:4429–4432.
- Xu Y and Szoka FC Jr (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**:5616–5623.
- Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, and Wilson JM (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci USA* **91**:4407–4411.
- Zabner J, Fasbender AJ, Moninger T, Poellinger KA, and Welsh MJ (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* **270**:18997–19007.
- Zerial M, and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* **2**:107–117.
- Zhou X, and Huang L (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim Biophys Acta* **1189**:195–203.
- Zuhorn IS, Kalicharan R, and Hoekstra D (2002) Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *J Biol Chem* **277**:18021–18028.