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Cellular Uptake and Intracellular Trafficking of Antisense and siRNA Oligonucleotides

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Abstract

Significant progress is being made concerning the development of oligonucleotides as therapeutic agents. Studies with antisense, siRNA, and other forms of oligonucleotides have shown promise in cellular and animal models and in some clinical studies. Nonetheless our understanding of how oligonucleotides function in cells and tissues is really quite limited. One major issue concerns the modes of uptake and intracellular trafficking of oligonucleotides, whether as 'free' molecules, or linked to various delivery moieties such as nanoparticles or targeting ligands. In this review we examine the recent literature on oligonucleotide internalization and subcellular trafficking in the context of current insights into the basic machinery for endocytosis and intracellular vesicular traffic.

Introduction

The concept of using oligonucleotides as therapeutic agents emerged more than three decades ago when antisense molecules were first described [1]. More recently enthusiasm for this approach was rekindled by the discovery of RNA interference [2] and of the ability to activate this process using exogenous short double stranded RNAs (siRNAs) [3]. The potential of oligonucleotide-based therapeutics has been further reinforced by new discoveries concerning the complex roles of non-coding RNAs in regulating many facets of genome function [4]. Currently multiple approaches are available for employing oligonucleotides to influence the extent and pattern of gene expression. This includes using conventional antisense or siRNA molecules to selectively degrade mRNA [5,6], antagomirs to block the actions of miRNAs [7], splice shifting oligonucleotides to alter gene expression patterns [8], decoys to block transcription factors [9], CpG rich oligonucleotides to stimulate the immune system [10], and triplex oligonucleotides for targeted mutagenesis [11]. Each of these approaches has benefited immensely from recent progress in oligonucleotide chemistry that has resulted in the creation of stable and potent molecules [12-14]. However, despite many research advances and the initiation of multiple clinical trials [5,15], the evolution of oligonucleotides as therapeutic agents has been hindered by the fact that delivery of these large, usually highly charged, molecules to their intracellular sites of action is a very challenging task [16,17]. In this article we review current information concerning the uptake of oligonucleotides and their intracellular trafficking. We consider the fate of 'free' oligonucleotides, of conjugates with various targeting ligands, and of oligonucleotides associated with nanoparticle carriers. The emphasis will be on events at the cellular and subcellular level rather than on whole animal pharmacokinetics and biodistribution, although some informative *in vivo* studies will be discussed. A theme that will pervade this article is the enormous complexity of the processes that govern intracellular traffic of internalized

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molecules and that thus influence the therapeutic potential of exogenously administered oligonucleotides.

1. Overview of Endocytotic and Trafficking Pathways

Although it is commonly accepted that antisense and siRNA oligonucleotides usually enter cells via endocytosis, this broad statement masks the complexities inherent in multiple pathways of internalization and subsequent intracellular trafficking. Here we will describe some of these events and discuss the implications for oligonucleotide pharmacology. We will also emphasize the intricate and highly selective protein machinery that underlies all aspects of endocytosis and trafficking.

a. Pathways of endocytosis and the associated protein machinery

Over the last few years it has become clear that there exist multiple pathways of endocytosis in addition to the archetypal clathrin coated pit pathway [18-20]. A diagram depicting some of these pathways is shown in Figure 1. In classical clathrin mediated endocytosis cell surface receptors and their associated ligands interact with adapter proteins including AP-2, and with a host of accessory factors, that cluster the receptors into specialized membrane areas subtended by a network of clathrin triskelions. The clathrin network, as well as specialized BAR domain proteins such as SNX9 and amphiphysin that sense and promote membrane curvature, contribute to invagination; this is followed by pinching off of a clathrin-coated vesicle through the agency of the dynamin GTPase [21]. The coated endosome quickly uncoats under the influence of a set of proteins including auxilin and hsc70 and now the uncoated vesicle is ready to begin its intracellular journey. Many important receptors and ligands are internalized via the clathrin pathway including LDL, transferrin, and many activated G Protein Coupled Receptors [20,22].

The caveolar pathway has also evoked a great deal of interest [23]. Many cells display small invaginations that are rich in cholesterol and sphingolipids and that contain caveolin1, a 21kD protein that inserts a hydrophobic hairpin into the membrane while both N- and C-termini are cytosolic. Additionally, the cavins, are coat proteins that help stabilize caveolar structures. There is some controversy as to whether caveolae generate independent intracellular vesicles or whether they remain as tubular structures linked to the plasma membrane; however, substantial evidence suggests that caveolae generate vesicles that can contribute to intracellular membrane traffic. In general caveolar structures are smaller (<100 nanometers) than other forms of endocytotic vesicles, which may reach several hundred nanometers in diameter. Cholera toxin (CTxB), SV40 virus, and GPI-linked membrane proteins are among the entities that are preferentially internalized via caveolae (however, some of these moieties are also internalized by other pathways). It seems likely that dynamin is involved in the disjunction of caveolae, but the evidence is not as clear as for the clathrin-mediated pathway. Many proteins in addition to caveolin have been observed to be associated with caveolae; in particular these structures are rich in molecules involved in signal transduction [24-26].

Recently a number of clathrin- and caveolin- independent pathways have been delineated to various degrees. These pathways are often defined either in terms of the morphologies of the vesicles they generate or in terms of the cargo that is preferentially internalized. For example, the flotillins are membrane-inserted proteins that may be involved in ordering lipid domains and subsequent endocytosis similarly to caveolin. Both CTxB and GPI-linked proteins have been associated with flotillin-rich membrane microdomains. Interestingly some reports suggest that dynamin is not needed for internalization of cargo via flotillin containing vesicles [27]. Thus the flotillin pathway represents one example of a clathrin and caveolin independent internalization mechanism.

Another key internalization mechanism is the CLIC/GEEC pathway that seems to be particularly important for fluid phase endocytosis [20]. The acronym is for Clathrin and Dynamin Independent Carriers (CLIC)/GPI-AP Enriched Early Endosomal Compartments (GEEC). This pathway gives rise to high volume tubular endosomes that are rich in GPI-proteins (including the folate receptor FRα) and that typically contain fluid phase markers such as dextrans. As the name implies, dynamin is not necessary for the pinching off of these vesicles. Instead membrane scission may be mediated by GRAF1, a BAR domain containing GTPase activating protein [28].

Additional clathrin and caveolin independent pathways exist [20,28]. This includes a pathway involved in the internalization of a form of the IL2-Receptor, certain potassium channels, and the FCεR1 immunoglobulin receptor. This pathway involves dynamin-mediated disjunction of vesicles from the plasma membrane; as well this pathway seems to be regulated by protein kinases of the PAK family. Another pathway that generates both vesicular and tubular structures has been implicated in the internalization of MHC class I histocompatibility proteins; a role for dynamin has not yet been found for this pathway.

Macropinocytosis describes a process by which cell protrusions pinch off large volumes of extracellular fluid and is thus an important aspect of fluid phase endocytosis [29]. It is also involved in the internalization of clustered, activated receptor tyrosine kinases. Generation of these relatively large structures involves the actinomyosin contractile machinery and is thus associated with typical regulators of such processes including the Rac GTPase and PAK family kinases, but probably not dynamin. Other large volume internalization mechanisms such as phagocytosis and entosis come into play in specialized cells or unusual circumstances but these are unlikely to play much of a role in the processing of oligonucleotides in the majority of cell types [20]. The actin cytoskeleton plays an important role in most of the endocytotic processes described above; however, not all internalizations require actin. Thus certain arenaviruses enter cells by a pathway that is independent of clathrin, caveolin, dynamin and actin [30]. Interestingly we recently found that phosphorothioate antisense oligonucleotides seem to enter cells by this pathway as well [31].

In summary, we know of multiple pathways for endocytosis with more probably remaining to be discovered. This provides daunting complexity but also exciting opportunities for oligonucleotide pharmacology. Thus by targeting antisense or siRNA to specific cell surface receptors it is possible to influence the initial route of internalization. As we will discuss below, this may have important implications for further processing and for the ultimate biological effect of the oligonucleotide.

b. Trafficking downstream of initial internalization- Rabs, Tethers and Snares

Whether presented in unmodified form, as a chemical conjugate, or associated with a nanocarrier, an oligonucleotide entering a cell in an endosome encounters a complex maze of intracellular pathways that can lead to many destinations and that is regulated by intricate protein machinery [32]. Key subcellular membrane bound compartments include early and recycling endosomes, late endosomes/multi-vesicular bodies, lysosomes, the Golgi apparatus and the endoplasmic reticulum. (see Figure 1). Trafficking is not a random process, but rather it is a carefully orchestrated ballet that allows the cell to transport endogenous and exogenous materials to the most appropriate place. Many pathogens have learned to exploit these events; for example certain viruses as well as many bacterial toxins engage the so-called retrograde transport process [33] and are thus delivered to the trans-Golgi compartment from which they seem to have ready access to the cytosol. Ultimately this is the goal of oligonucleotide delivery as well, to leave membrane bound compartments and access the cytosol and nucleus. It seems increasingly clear that appropriate manipulation of endocytotic and trafficking pathways can help attain this goal. For example, inhibition of

intracellular protein kinase A activity with small molecule drugs can prevent trafficking into the late endosomal/lysosomal compartments in cellular delivery of nucleic acids via polyplexes and lipoplexes, thus improving the transfection efficiency [34].

We only partially understand how endocytotic cargos are delivered to particular subcellular compartments. Many of the internalization pathways described above converge at the stage of early endosomes. This raises the question of how receptors, ligands and cargo that have been internalized by different initial endocytotic mechanisms traffic to different subcellular destinations. Recent evidence suggests that membrane domains originating from different internalization pathways maintain their identity within early endosomes, thus setting the stage for specific sorting and trafficking to distinct downstream destinations [28].

The past few years have seen enormous progress in understanding the molecular mechanisms of intracellular trafficking. All membrane traffic proceeds by the same basic steps: (a) a coated vesicle is pinched off from a larger donor membrane compartment; (b) the vesicle uncoats allowing the display of tethering and fusion proteins; (c) the vesicle is carried to its destination along ‘tracks’ provided by actin- or tubulin- based cytoskeletal structures; (d) the vesicle recognizes its target membrane compartment using tethering proteins and then utilizes SNARE proteins to complete the fusion process and deliver membrane and contents to the target compartment [32]. A simplified diagram of this process is shown in Figure 2. There are numerous variations of the overall theme and still some uncertainty about the exact sequence of events, but the overall picture is quite clear.

For a more detailed discussion of intracellular trafficking mechanisms it is necessary to introduce the Rab proteins. The Rabs comprise a large (>60) family of GTPases that serve as molecular switches to regulate multiple aspects of intracellular vesicular traffic [35,36]. In their active GTP loaded form Rabs bind to and modulate the function of downstream effector proteins. In their inactive GDP loaded form they associate with Rab-GDI (Rab GDP Dissociation Inhibitor) proteins that both stabilize the inactive form and serve as chaperones to regulate the balance between cytosolic and membrane bound Rab. As with other small GTPases, the equilibrium between active and inactive forms is regulated by Rab specific GEFs (Guanine Nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins).

Rabs serve many different roles in regulating intracellular trafficking including vesicle uncoating, vesicle movement along cytoskeletal tracks, and the ultimate fusion events. For example, Rab5 regulates the uncoating of clathrin-coated vesicles during classical receptor mediated endocytosis while Rab 27A links melanosomes to the myosin Va motor protein via a Rab-binding adaptor protein. Rabs also associate with tethering proteins, a good example being the association of Rab1 with Golgin-type tethers. Because of their high specificity Rab proteins serve as excellent markers of individual membrane trafficking pathways. Thus Rab5 is associated with the movement of clathrin coated vesicles to the early endosome, Rab 4 with the early endosome and recycling to the plasma membrane, Rab7 with late endosomes/lysosomes, Rab9 with late endosome to Golgi traffic, and Rab11 with a slowly recycling perinuclear endomembrane compartment (see Figure 1). However, a complex aspect of Rab function concerns the formation of ‘Rab domains’ within particular endosomes. Thus studies using low levels of expressed green fluorescent protein-Rab chimeras, have shown that different Rab proteins localized on the same organelle can occupy distinct membrane microdomains [35]. This segregation of Rabs may play a role in the endosomal sorting processes discussed above [28].

As mentioned above, the initial event in intracellular trafficking is the pinching off of a coated vesicle from a donor membrane. The formation of clathrin coated vesicles (CCVs) at the plasma membrane is a good example, but other types of coats exist such as the COPI and

COPII coats of the Golgi and ER. Disjunction of the coated vesicle is accomplished by dynamin in the case of CCVs, but by other mechanisms as well. For example, budding off of vesicles from late endosomes to the trans-Golgi is accomplished by the retromer complex with its BAR-domain containing SNX proteins [37].

Tethering proteins impart selectivity to vesicle traffic by supporting preferential interactions between the vesicle and its ultimate target membrane compartment. There are two broad classes of tethering molecules; the coiled-coil tethers such as the Golgins and the multi-subunit tethers [38]. Tethers are thought to bridge membranes and promote fusion by binding to both Rab proteins and to SNARES. There is some uncertainty about when and how tethering proteins associate with trafficking vesicles, particularly whether tethers interact with vesicle coat proteins or if tethering takes place after uncoating [32,35].

The ultimate transfer of both the membrane material and the contents of the shuttle vesicle to the recipient compartment is accomplished via a fusion process mediated by SNAREs (soluble N-methylmaleimide sensitive factor attachment protein receptors) [39]. After recognition is accomplished by tethering proteins, SNARES on the vesicle (v-SNARES) interact with SNARES on the target membrane (t-SNARES) to form a four-helix bundle that undergoes conformational change to induce membrane fusion. There is considerable specificity in this process since only specific cognate pairs of v- and t-SNARES will sustain fusion. Resegregation of the v-SNARES and t-SNARES is mediated by the ATP-dependent NSF/SNAP protein complex.

c. Escape from endosomes during intracellular trafficking

The complex machinery described above is designed to move endogenous molecules to specific membrane destinations within the cell. Although it is quite efficient and selective, nonetheless there is some opportunity for molecules to escape from endomembrane compartments to the cytosol. Intracellular trafficking involves a highly dynamic flux of membrane vesicles that engage in a multitude of fusion and disjunction events. In recent years fusion mechanisms in both natural membranes and artificial lipid membranes have been studied in great detail [40,41]. While this literature is largely beyond the scope of this review, there are a few key points to be noted. First, fusion involves localized stress on the fusion partners including the formation of non-bilayer lipid domains [40,42]. Second, there is good evidence that non-bilayer regions of membranes can be much leakier than bilayer regions [43,44]. Third, many enveloped viruses fuse with cells via specialized membrane interacting proteins that, while differing in sequence, act in a manner similar to cellular SNARE proteins; the influenza virus fusion protein is a good example [45]. In many cases these proteins can also induce increases in membrane permeability [46]. Therefore there is an intrinsic relationship between the fusion events inherent in intracellular trafficking and transient leakage of vesicular contents (see Figure 2). Thus the innate activity of oligonucleotides taken up by cells is likely due to a modest amount of continuous leakage from endomembrane compartments that spontaneously occurs during intracellular trafficking, while much of the current technology for enhancing oligonucleotide effects is aimed at increasing the extent of endosomal release, as discussed below.

d. Nuclear entry

Antisense oligonucleotides encounter their pharmacological targets in the nucleus, but nuclear entry may not be the rate-limiting step for oligonucleotide pharmacology. Studies have shown that oligonucleotides, particularly those with phosphorothioate backbones, are able to continuously shuttle between the nucleus and the cytoplasm. This is an active process mediated by nuclear pore structures; however, it does not require classical nuclear

localization signals. For conventional phosphodiester oligonucleotides both passive diffusion and active transport have been described as nuclear entry mechanisms [47,48].

2. Uptake and Trafficking of 'Free' Oligonucleotides

There has been considerable work done trying to understand the cellular uptake, trafficking and tissue distribution of oligonucleotides themselves, absent any specific targeting or carrier mechanisms. Some of the earlier literature has been covered elsewhere [49] and more recent reviews have updated information on the pharmacokinetics and biodistribution of antisense and siRNA [5,50]. While uncharged oligonucleotides such as morpholino and peptide nucleic acid derivatives, as well as most forms of siRNA, are rapidly excreted via the kidney, phosphorothioate (PS) oligonucleotides display substantial binding to plasma proteins and cells and are thus retained in the body for longer periods. Preferential *in vivo* uptake by certain cell types, particularly kidney proximal tubule cells and liver Kupffer cells, has been noted both for PS antisense compounds and for siRNA [51,52]. Recent publications have provided new insights into the uptake and trafficking of 'free' or 'naked' oligonucleotides. Thus a study using phosphorothioate antisense oligonucleotides in a transformed liver cell line and in murine livers has suggested the co-existence of productive and non-productive routes of uptake [53]. The non-productive pathway seems to involve trafficking to lysosomes, while the pathway that results in RNase H dependent antisense effects involves trafficking that eventually leads to interaction with cellular pre-mRNA. A somewhat confusing observation is that the productive pathway is not blocked by siRNA targeting clathrin but is blocked by siRNA targeting AP2M1 an adapter protein in the clathrin endocytotic pathway. In any case this study provides one of the most detailed accounts to date of the subcellular fate of free oligonucleotides in cells and tissues. Other interesting studies, both in cell culture and in mouse models, have involved the so-called 'gymnotic' uptake of antisense oligonucleotides modified with LNA (locked nucleic acid) moieties [54,55]. In contrast with the previously mentioned study, which used nanomolar concentration of PS oligonucleotides, the antisense effects of 'naked' LNA usually required micromolar concentrations. Subcellular distribution studies surprisingly suggested that the deoxy LNA compounds became associated with P-bodies that are usually thought to be sites of siRNA action [54].

There have been many attempts to identify endogenous receptors for antisense or siRNA molecules; however, much of this literature is problematic and no direct evidence for their involvement in oligonucleotide trafficking has been provided. Integrins of the beta2 subclass [56] as well as scavenger receptors [51] have been suggested as candidates, but this is controversial [57]. A putative oligonucleotide transporter has also been described [58,59] but there has not been a great deal of confirmatory work on this finding by other groups. Another interesting candidate is the mammalian homolog of the double-stranded RNA (dsRNA) transport protein SID-1 found in *Caenorhabditis elegans* [60]. SID-1 clearly plays a key role in the spread of RNA interference from cell to cell in some invertebrates. However, initial reports of a role for SID-1 in uptake of siRNA by mammalian cells [61-63] have not been followed by more advanced mechanistic studies. An interesting variant on oligonucleotide delivery involves the stimulation of siRNA uptake by phosphorothioate oligonucleotides [64]; the phenomenon seems clear although the underlying mechanism is still not fully resolved.

The most convincing examples of cellular receptors for oligonucleotides involve the Toll-Like Receptor (TLR) family [65,66]. As a simplistic summary, TLR9 binds DNA having CpG motifs, TLRs7/8 bind single stranded RNA, while TLR3 binds double stranded RNA. Although these TLRs are usually found within endosomes rather than at the cell surface, in some cases they seem to be able to assist in the accumulation of oligonucleotides by cells.

For example, as discussed in more detail below, a CpG oligonucleotide was able to substantially enhance the cell uptake and effect of a conjugated siRNA [67].

In summary, at this point there is only a limited amount of information about the mechanisms involved in cell uptake and subcellular trafficking of 'free' oligonucleotides, with many contradictions needing to be resolved. This seems an important topic to address since most of the clinical studies with antisense or siRNA to date have used 'free' compounds.

3. Uptake and Trafficking of Conjugates and Complexes of Oligonucleotides with Cell Penetrating Peptides

Over the last decade there has been substantial interest in using so called 'cell penetrating peptides' (CPPs) for the delivery of oligonucleotides. CPPs (sometimes called 'protein transduction domains') are peptides, usually rich in cationic residues, that purportedly have the ability to cross membranes and in doing so convey attached cargoes into the cytosol. The TAT and Antennepedia peptides are the archetypal forms, but a large variety of new CPPs have been described more recently [68-70]. Originally it was thought the CPPs could directly translocate across the plasma membrane (and some recent publications still support this view [71,72]). However, most reports suggest that cationic CPPs bind initially to negatively charged proteoglycans at the cell surface, are internalized into endosomes, and may then escape from those structures. Studies have linked the entry pathway of TAT or its conjugates to clathrin-mediated endocytosis [73], macropinocytosis [74], or calveolar endocytosis [75] in various cell types and experimental circumstances. In any case, it is clear that the nature of the attached cargo plays a major role in CPP uptake mechanisms and in the effectiveness of cytosolic delivery [76,77]. In general, as might be anticipated, smaller cargos are delivered more effectively; for example, TAT-containing proteins are mostly trapped in cytoplasmic vesicles after cellular uptake, while the TAT-peptide conjugates distribute throughout the cell [76].

There have been numerous studies of both chemical conjugates and noncovalent complexes of antisense and siRNA with CPPs. Work on the chemical conjugates has been the subject of recent reviews [78,79]. In general, conjugates of CPPs with charged oligonucleotides have not proven to be very promising, while conjugates with uncharged oligonucleotides such as morpholinos or peptide nucleic acids (PNAs) have displayed more activity. This may be of particular interest in the context of splice-shifting oligonucleotides (SSOs) that can be used to correct or modify RNA splicing processes [8]. Recent work with conjugates of novel CPPs to PNA [80] or morpholino [81] SSOs have shown good splice-correction results in cell culture, and promising therapeutic performance in mouse models of Duchenne muscular dystrophy [82,83]. Another interesting approach has been to make CPP-oligonucleotide conjugates that also contain a lipid moiety [84]; this seems to lead to enhanced endosomal escape and thus greater efficacy of the conjugated PNA SSO.

Despite some early studies to the contrary, chemical conjugation of classic antisense or siRNA oligonucleotides to CPPs has been problematic. For this reason a number of laboratories have turned to non-covalent complexation of CPPs with anionic oligonucleotides to form various nanoparticles. This strategy has been the subject of recent reviews [85,86]. The complexation of cationic CPPs with oligonucleotides results in the formation of nanoscale particles [87-89]. These entities can be very effective in delivering oligonucleotides; for example siRNA complexed with a CPP modified with a lysosomotropic chloroquine analog provided strong 'knockdown' in a number of difficult to transfect cell lines [90]. SSOs complexed with PepFect 14, a new stearylated CPP, induced higher splicing correction than the commercially available lipid-based vector Lipofectamine

2000, and the CPP/SSO complexes could be incorporated into solid dispersions so that the stability was improved significantly [89]. CPP/oligonucleotide complexes have also been tested *in vivo* [87,90]. However, there are some obvious concerns about systemic administration of such nanoparticles in terms of rapid clearance by the reticuloendothelial system, limited biodistribution, and toxicity [17]. Perhaps the proponents of CPP/oligonucleotide complexes can adapt approaches from other nanoparticle technologies, such as use of PEG to improve biodistribution characteristics.

There has been only limited investigation of basic uptake and trafficking mechanisms of CPP oligonucleotide conjugates or complexes. One type of CPP/siRNA complex has received extensive biophysical analysis leading to the suggestion that these complexes can enter cells by non-endocytotic means [85]; however, physical analysis may offer only limited insights into cellular mechanisms. Other studies of these complexes using pharmacological inhibitors also suggested non-endocytotic uptake pathways [91]. In contrast, a recent study using complexes of novel CPPs with 2'-O-Methy-phosphorothioate splice switching oligonucleotides presents a very different picture, with the initial uptake occurring mainly by clathrin-mediated endocytosis as discerned by using pharmacological inhibitors of uptake pathways [92]. The effectiveness of the splice correction was not dependent on total uptake but rather on increased release of the SSO from endosomes. There have been some relatively recent mechanistic studies of chemical conjugates of CPPs with SSOs [80] also suggesting cell entry via clathrin-mediated endocytosis. Unfortunately most of the studies of complexes or conjugates of CPPs with oligonucleotides have relied on use of rather non-specific pharmacological inhibitors to evaluate uptake mechanisms. While this is a useful first step, such studies should be followed up by further analysis using molecular means to alter uptake and trafficking pathways, and by more detailed studies of co-localization with known markers of intracellular membrane compartments.

4. Uptake and Trafficking of Receptor Targeted Ligand-Oligonucleotide Conjugates

In addition to the CPP-oligonucleotide conjugates described above, there has been substantial work involving other covalent modifications of antisense and siRNA to enhance their biological properties, as summarized in several recent reviews [93-96]. Much of this has focused on conjugation with cholesterol or other lipophilic moieties to increase oligonucleotide lifetime in the circulation and to promote uptake via lipoprotein receptors in the liver and elsewhere [7,63,97]. By contrast there is a relative paucity of reports concerning the synthesis of monomolecular conjugates of oligonucleotides with ligands designed to target specific cell surface receptors. A few early studies on this topic involved folate-conjugated antisense oligonucleotides that associated with the FRα receptor [98], N-acetyl galactosamine conjugates that targeted the hepatic asialoglyoprotein receptor [99], and conjugates of siRNA with Insulin-Like Growth Factor 1 for targeting its cognate receptor [100].

More recently there has been a substantial increase in work on receptor targeting of oligonucleotides. This includes our own reports on RGD peptide conjugates of splice switching antisense oligonucleotides that can be delivered to melanoma cells via the αvβ3 integrin [31,101], as well as bombesin conjugates that are targeted to prostate cancer cells via the BB2 receptor, a member of the G Protein-Coupled Receptor (GPCR) superfamily [102]. We have also targeted oligonucleotides to tumor cells using anisamide, a high affinity small molecule ligand for the sigma receptor [103]. Another interesting approach is the delivery of siRNA by targeting TLRs [67]. Thus an un-methylated CpG oligonucleotide known to bind to TLR9 was chemically conjugated to a siRNA. This resulted in enhanced uptake by dendritic cells, macrophages and B-cells, all known to express TLR9, as well as 'knockdown' of

endogenous and reporter genes. Use *in vivo* of a CpG siRNA targeting the immunosuppressive regulator Stat3 resulted in enhanced antitumor immune responses. Another very promising approach to receptor specific delivery of oligonucleotides involves use of nucleic acid aptamers [104]. A pioneering report described the characterization of chimeric oligonucleotides comprised of an aptamer that bound with high affinity to the PMSA receptor in prostate cancer cells and siRNAs that affected key survival genes such as *Plk1* and *Bcl2* [105]. These conjugates were taken up selectively by cells that expressed PSMA receptor, were effective at 'knockdown' of the target messages in cell culture, and displayed antitumor activity when locally administered. More recently a chemically optimized version of a PMSA aptamer-Plk1siRNA chimera displayed antitumor activity against PMSA expressing tumors when given by systemic administration [106]. In another impressive study, similar aptamer-siRNA chimeras inhibited tumor growth *in vivo* using siRNAs directed against *Upf2* and *Smg1*, two genes involved in nonsense mediate mRNA decay and thus in immune regulation of tumors [107]. Other important examples of aptamer-siRNAchimeras are also beginning to emerge [108]. These various studies have validated the concept that monomeric ligand-oligonucleotide conjugates can produce significant pharmacological effects both in cell culture and in animals, in the absence of any transfection agents. A recent review has provided an extensive overview of receptor targeting of oligonucleotides [109].

Recent work on receptor-targeted oligonucleotide conjugates has also provided an important new insight for the problem of effective delivery of antisense and siRNA molecules. The essence is that the initial route of uptake (and subsequent trafficking) plays an important role in determining the pharmacological effectiveness of the oligonucleotide. Thus in our studies comparing effects of 'free' or receptor targeted SSOs, we consistently noted that the targeted conjugates were more effective in attaining splice correction, even when the targeted and free oligonucleotide were accumulated to the same level in cells [31,101,102]. Studies with the CpG siRNA conjugate revealed that the presence of TLR9 was critical for attaining effective 'knockdown' even though cells lacking the TLR could still take up the conjugate [67]. This also suggests a receptor specific aspect to intracellular trafficking and biological outcome. These observations also harmonize with recent cell culture and *in vivo* studies of free phosphorothioate oligonucleotides that suggest the co-existence of productive and non-productive routes of internalization [53,110]. These various observations support the concept that it may be possible to optimize the effectiveness of an antisense or siRNA molecule by controlling its pathway of uptake and intracellular trafficking, without resorting to harsh cationic lipid or cationic polymer transfection agents.

Consequently, investigators interested in the design of new receptor-targeted oligonucleotide conjugates should consider several basic issues. First, and most obvious, the ligand must display high affinity for the receptor, preferably in the low nanomolar range. Second, in order to achieve cell-type specific delivery there must be differential expression of the receptor in the cell of interest as compared to other cell types. An initial estimate of relative receptor expression in various tissues or between tumors and normal tissue can be gleaned from contemporary gene expression data bases, at least at the mRNA level (for example, http://www.ebi.ac.uk/gxa/). Third, in order to deliver sufficient quantities of oligonucleotide, the receptor of interest must be relatively abundant. The abundance of various receptors can range from a few copies per cell up to hundreds of thousands of copies. However, there are relatively few compact sources for this type of information at the protein level, and thus the literature must be searched for values for specific receptors. Fourth, the receptor of interest must cycle efficiently between the plasma membrane and endosomes. There is information in the literature on the propensity of various families of receptors to be internalized and recycle. For example, different members of the integrin family can be internalized via caveolae or via coated pits and be recycled via Rab4- or Rab11-dependent trafficking processes [111]. Agonist binding can stimulate the internalization of GPCRs via clathrin-

coated vesicles and their ultimate recycling to the plasma membrane or sorting to multivesicular bodies for degradation in lysosomes [112]. Receptor tyrosine kinases of the EGF-R family also respond to agonist binding by internalization via the clathrin pathway followed by trafficking to multivesicular bodies and lysosomes [113]. Another important consideration is the point at which the receptor-targeted oligonucleotide ceases to parallel the intracellular trafficking of the receptor itself and enters separate pathways. It is not yet clear which pathway of internalization and trafficking will be optimal for oligonucleotide delivery, but it is clear that trafficking pathways must be taken into account. Thus the design of novel targeted oligonucleotide conjugates should start with a clear picture of the underlying receptor biology in order to have a reasonable chance of success.

5. Uptake and Trafficking of Oligonucleotides Associated with Nanocarriers

The most popular approach to enhancing delivery of antisense, siRNA or other types of oligonucleotides is to incorporate the nucleic acid into some form of nanoparticle, with the intent of overcoming biological barriers and increasing both cell uptake and escape from membrane compartments [16,17,114,115]. Lipid based carriers have proven to be very efficacious for the delivery of siRNA to the liver [116], while a variety of polymeric nanoparticles [117] and other types of nanocarriers [118] have also been developed for siRNA delivery. Functional delivery of siRNA to tumors has been challenging, but recently targeted lipid based nanoparticles have displayed substantial activity in this context [119]. Nonetheless, there remain concerns about possible toxicities associated with the cationic polymers or lipids most commonly used to form nanocarriers for oligonucleotide delivery [120,121]. Since there is such a vast literature on use of nanoparticles as oligonucleotide delivery agents, we will focus our discussion on a relatively few reports that have mechanistically addressed issues of cellular uptake and trafficking.

a. Uptake and trafficking of non-targeted oligonucleotide nanocarriers

A very interesting report has challenged the conventional view that cationic lipid carriers functionally deliver siRNA via endocytosis followed by escape from endosomes [122]. This group found that while much of the lipid and siRNA did enter cells by some form of endocytosis, it was only a minor component of the cell-associated siRNA that contributed to ‘knock down’ function and that this component probably came from fusion between the siRNA lipoplexes and the plasma membrane. This study is notable for going beyond simplistic use of chemical inhibitors and employing molecular reagents such as dominant negative versions of dynamin and caveolin to probe uptake pathways. Another interesting recent study followed the uptake and trafficking of siRNA associated with perfluorocarbon nanoparticles [123], finding that delivery was via formation of cell-nanoparticle hemifusion complexes followed by lipid raft mediated internalization. This study made good use of the strategy of co-localization with markers that are known to be internalized via particular pathways. A recent investigation from our laboratory compared the uptake and trafficking pathways of splice switching antisense oligonucleotides as delivered via cationic lipids or via PEI, a cationic polymer [124]. Several strategies including pharmacological inhibitors, co-localization with known markers of internalization, as well as use of molecular reagents were employed in this study. Interestingly, in agreement with the study on siRNA delivery discussed above, functional delivery of antisense associated with lipoplexes was apparently due to fusion at the plasma membrane, while delivery via polyplexes took place through an unconventional form of endocytosis. Thus even with widely utilized transfection agents there seems to be a diversity of delivery mechanisms that need to be better understood.

b. Uptake and trafficking of targeted oligonucleotide nanocarriers

Over the last few years several very interesting forms of targeted nanoparticles have been developed for purposes of siRNA delivery and some very impressive functional results have been attained. For a review of some of the early literature see [79,125]. However, there is a dearth of information about the mechanistic aspects of the interplay of these particles with cells. For example, some very high profile publications have described a strategy where a chimeric polypeptide is formed between a targeting moiety and a cationic poly-arginine sequence; the cationic sequence is used to complex siRNA while the targeting moiety provides receptor selective delivery. Thus, a chimera including a rabies virus peptide that binds an acetylcholine receptor was used to target siRNA to neuronal cells and demonstrated therapeutic effects in a mouse model of viral encephalitis [126]. In another study a single chain antibody to the T cell protein CD7 was used as the targeting moiety and a siRNA cocktail to CCR5 and to viral genes was used to inhibit HIV infection [127]. This same general approach has also been used to modulate inflammatory conditions, and the growth of other viruses [128,129]. However, these various studies provide no information on the mechanism of cellular uptake and subsequent trafficking of these interesting chimeric complexes. A somewhat similar strategy involved complexation of siRNA to chimeric fusion proteins that included a single chain antibody as the targeting moiety and a segment of protamine to bind the nucleic acid. This strategy has been used in models of HIV and leukocyte activation [130,131]. However, despite very interesting functional data, once again there is little information on the mechanism of delivery. Another interesting approach involves preparation of a complex polymer that includes covalently attached siRNA, PEG, and a N-acetylgalactosamine targeting ligand for interaction with the liver asialoglycoprotein receptor [132]. Similarly lactosylated PEG-siRNA conjugates formed into polyplexes have been used to target hepatic carcinoma models [133]. However, no substantial information on trafficking is available concerning these hepatic targeting approaches. Lipid nanoparticles incorporating anisamide, a small molecule ligand for the sigma receptor, have shown promising effects in several animal tumor models [134,135] but only limited information is available concerning uptake and trafficking. One type of targeted siRNA nanoparticle has already been tested in the clinic in patients with solid tumors [136]. The formulation is comprised of siRNA against ribonucleotide reductase, a cationic cyclodextrin-containing polymer, polyethylene glycol, and human transferrin as a targeting ligand. While some work has been done concerning the cellular uptake [137] and overall biodistribution and tumor uptake of these materials [138], there are few details concerning their intracellular trafficking. Thus for targeted nanoparticles containing oligonucleotides there is a dearth of information concerning the mechanistic details of the uptake and trafficking processes. Studies of this type could help to make the future development of these materials more efficient

Conclusions

The thrust to develop antisense, siRNA, and other types of oligonucleotides as therapeutic agents has, to some degree, outstripped our fundamental knowledge of how these molecules behave in cells and in the body. While extensive information exists regarding the overall pharmacokinetics and tissue distribution of both classic antisense and siRNA, we lack an equivalent depth of knowledge about behavior at the cellular and intracellular level. This deficit applies both to oligonucleotides administered in ‘free’ or ‘naked’ form and to these molecules when incorporated into nanocarriers or conjugated to targeting ligands. Recent work has provided some interesting and possibly surprising observations concerning the uptake and intracellular trafficking of oligonucleotides. Thus conventional phosphorothioate antisense molecules, which have been studied for years, turn out to have an unusual uptake mechanism that involves both a productive and a less productive path to nuclear sites of action. Work from our laboratory and from others has shown that the pharmacological

effectiveness of an antisense or siRNA can strongly depend on its route of uptake and trafficking. In particular, certain receptor-mediated processes seem to support productive delivery. As the oligonucleotide therapeutics field matures, and particularly as investigators seek to enhance specificity through targeted delivery, it will be important to employ basic cell biological principles in the design of delivery approaches. This includes identification of target receptors that are abundant, are differentially expressed, and are strongly linked to endocytotic pathways. Further insights into the intracellular trafficking of oligonucleotides will also facilitate the design of effective delivery systems.

Acknowledgments

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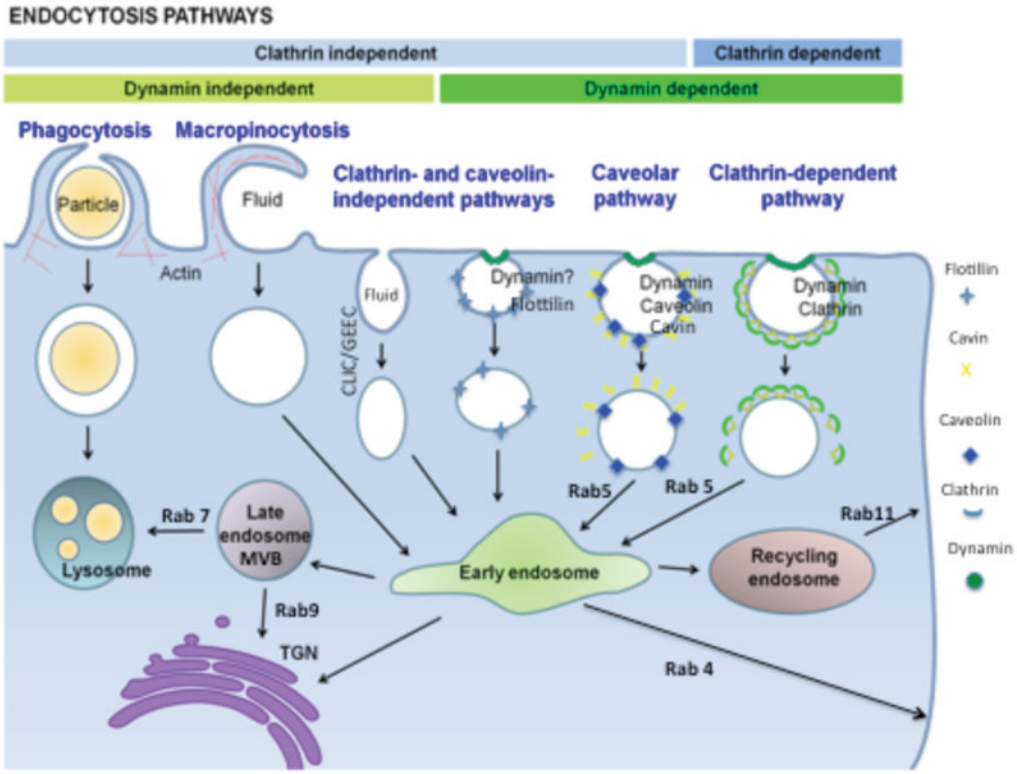

Figure 1. Pathways of Endocytosis and Trafficking

The figure illustrates several of the major internalization pathways discussed in the text. Phagocytosis takes places only in specialized cells such as macrophages and granulocytes while the other pathways are found in many cell types. A few of the key proteins involved in some of the pathways are indicated; however many other proteins that play a role are not depicted. Some aspects of the intracellular vesicular trafficking between various endomembrane compartments are also illustrated. Specific Rab GTPases play key roles controlling the flow of shuttle vesicles between individual compartments.

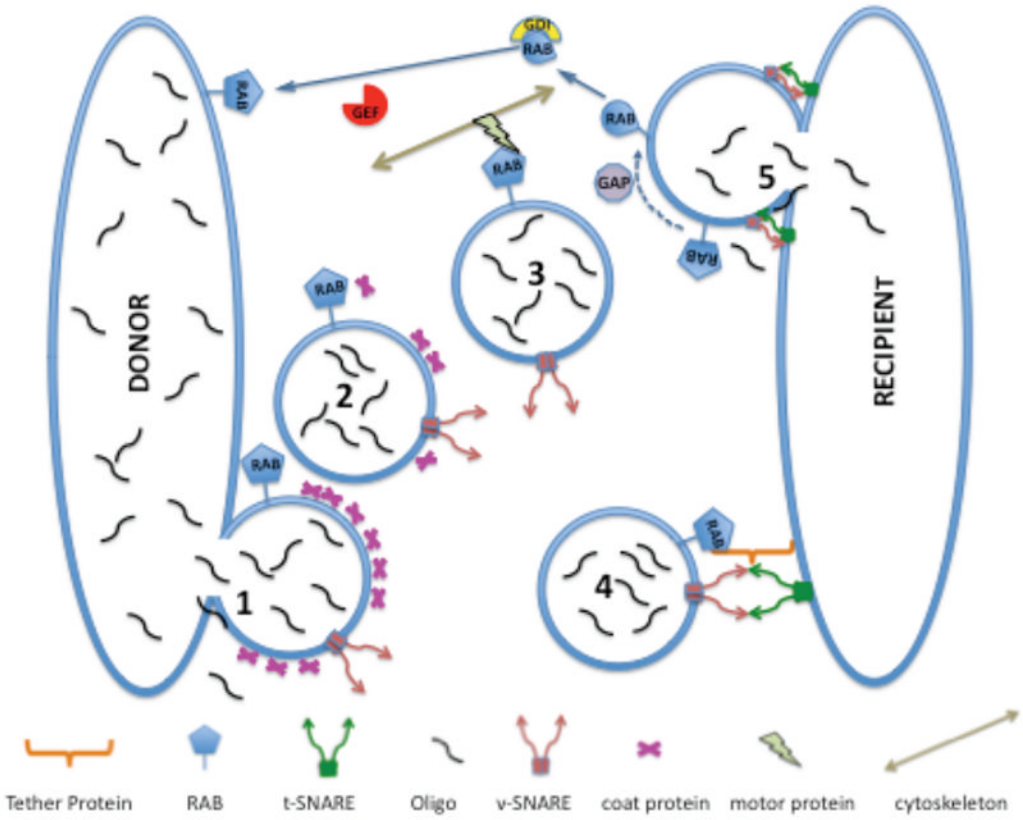

Figure 2. Proposed Mechanism of Vesicular Trafficking of Oligonucleotides

Oligonucleotides are initially accumulated in an endomembrane compartment (the DONOR compartment, for example, early endosomes) and are then trafficked by means of shuttle vesicles to various other endomembrane compartments (the RECIPIENT compartment, for example, the trans-Golgi). The first step (**1**) involves disjunction ('pinching off') of a shuttle vesicle under the influence of a coat protein as well as other accessory proteins. At this stage there are non-bilayer regions at the junction between the membranes of the DONOR compartment and the shuttle vesicle. This provides an opportunity for some oligonucleotide to escape to the cytosol. Step **2** involves uncoating of the coated vesicle; Rab proteins can contribute to this step. Step **3** comprises movement of the shuttle vesicle toward its destination along cytoskeletal tracks. Motor proteins such as various myosins (for the actin system) or dyneins or kinesins (for the microtubular system) propel the vesicle. Rab proteins are involved in forming the appropriate linkages to the cytoskeleton. Step **4** entails recognition of the RECIPIENT ('target') compartment by the shuttle vesicle. Tether proteins work with Rab proteins to provide interaction specificity while v-SNARE proteins in the vesicle membrane interact with t-SNARE proteins in the RECIPIENT compartment membrane to provide firm bridging, as well as contributing to specificity. In step **5** the SNARE proteins undergo major conformational changes, and with the assistance of accessory proteins, trigger fusion of the shuttle vesicle membrane with the membrane of the RECIPIENT compartment. At this stage non-bilayer regions exist at the junction between shuttle and RECIPIENT membranes potentially allowing escape of oligonucleotide.