Antisense c-*myc* Oligodeoxyribonucleotide Cellular Uptake

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Antisense oligonucleotides have therapeutic potential as inhibitors of gene expression. However, the mechanism by which an intact oligonucleotide reaches the intracellular site of action is unknown. In this study, we use an oligodeoxyribonucleotide 21-mer complementary to the translation initiation codon of the c-*myc* proto-oncogene to study the mechanism of oligonucleotide uptake and internalization into Rauscher Red 5-1.5 cells. We find trypsin-sensitive and trypsin-insensitive surface binding, in addition to internalization. Uptake is partially energy dependent and inhibited by charged molecules, including DNA, ATP, a random sequence oligonucleotide, and dextran sulfate. Uptake does not appear to occur via a traditional receptor-mediated uptake pathway because chloroquine, monensin, and phenylarsine oxide pretreatment does not significantly decrease internalization. An anion channel inhibitor, SITS, and the salts, NaCl, Na_2SO_4, and NH_4Cl, significantly decrease oligonucleotide uptake. Whether uptake occurs via a channel or a novel uptake mechanism is still unknown. A model is proposed which reasonably simulates the experimental data.

KEY WORDS: antisense; oligonucleotide; uptake; c-*myc;* channels; endocytosis.

INTRODUCTION

The specificity of oligonucleotide binding to target genes can make these compounds potent inhibitors of gene expression. They have potential for use as therapeutic agents in several areas including anticancer and antiviral chemotherapy. Because oligonucleotides are bulky and charged, the cell membrane is expected to pose an obstacle to entry. Initially, studies have been undertaken with the assumption that oligonucleotides cross cell membranes by passive diffusion (1). More recently the role of receptor-mediated uptake has been studied and a putative receptor protein has been identified (2,3). Our goal is to understand better the pathways by which oligodeoxyribonucleotides enter cells in the absence of drug delivery systems. In this study, we examine the uptake of ^{32}P-labeled antisense c-*myc* oligodeoxyribonucleotide 21-mer into Rauscher cells in the presence or absence of various inhibitors. We find that uptake involves greater than one process, is partially dependent on a trypsin-sensitive process, and is partially energy dependent. Uptake is not significantly influenced by pretreatment with several receptor-mediated endocytosis inhibitors but is reduced by the addition of charged compounds. The addition of either an anionic channel inhibitor, chloride or sulfate partially inhibits oligonucleotide uptake.

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MATERIALS

Antisense oligonucleotide 21-mer (5′-GAAGTTCACG-TTGAGGGG CAT), complementary to the mRNA of the c-*myc* proto-oncogene starting at the AUG translation initiation codon, and a nonsense oligonucleotide, a scrambled sequence of the antisense c-*myc* oligonucleotide, (5′-ATGGA-GTCACGTAACGGTTGG), were synthesized and purified by the Biomolecular Resource Center, UCSF, San Francisco, CA. The random sequence 21-mer (5′-CTAAAAGGG-CAGAAGAAGGAC) was obtained from the V.A. Genetics Core Facility, V.A. Medical Center, San Francisco, CA. The NH_4Cl was obtained from Baker, Phillipsburg, NJ; chloroquine, 2-deoxyglucose, SITS (4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid), salmon sperm nuclei, acrylamide, bis(*N,N′*-methylene-bis-acrylamide), phenylarsine oxide, and dibutyl phthalate were from Sigma Chemicals, St. Louis, MO; monensin was from Calbiochem, La Jolla, CA; and azide, trypsin, sodium dodecyl sulfate, EDTA (ethylenediaminetetraacetic acid), tris(hydroxymethyl)aminomethane, urea, boric acid, formamide, bromophenol blue, ACS II, and Scintiverse II were from Fisher Scientific, Fairlawn, NJ. Dionyl phthalate and X-Omat AR film were obtained from Kodak, Rochester, NY. [γ-^{32}P]ATP (3000 Ci/mol), [^{3}H]H_2O, [^{14}C]sucrose, and T4 kinase were obtained from Amersham, Arlington Heights, IL; and dextran sulfate was from Pharmacia, Uppsala, Sweden. Fetal calf serum was purchased from Hyclone, Logan, UT; penicillin and streptomycin were from UCSF Cell Culture Facility, San Francisco, CA; and minimal essential medium-alpha was from GIBCO, Gaithersburg, MD. Chrono-Lume reagent was obtained from Chrono-Log, Havertown, PA.

METHODS

The antisense 21-mer was labeled using T4 kinase and [γ-^{32}P]ATP to yield an external 5′ phosphate (4). Rauscher Red 5-1.5 cells (5) were maintained in minimal essential medium-alpha with 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal calf serum (preheated at 56°C for 1 hr). To confirm the biological activity of the antisense c-*myc* 21-mer at a 0.1 μM final concentration, cell concentrations were measured in 3-day proliferation assays.

Cells were centrifuged in 250-ml tubes at 600*g* and 25°C for 10 min and then resuspended in serum-free medium to a final concentration of 16–20 $\times$ 10^6 cells/ml. Cells were then equilibrated in 3-ml aliquots at 4, 15, 25, or 37°C for 5–10 min for temperature assays or pretreated at 37°C with 10 mM azide, 50 mM 2-deoxyglucose, or 0.25% trypsin for 60 min in noncompetitive inhibition studies. In competitive inhibition studies, 10 μM salmon sperm DNA, ATP, random-sequence oligonucleotide, or dextran sulfate was preincubated at 37°C with cells for 1–4 min. Salmon sperm DNA was extracted as described by Maniatis and co-workers (4). In anion inhibi-

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tion experiments, the cells were preincubated at 37°C for 15 min with 10 m*M* NaCl, NH_4Cl, or Na_2SO_4 or for 60 min with 60 μ*M* SITS. Cells were pretreated at 37°C for 30 min with 10 m*M* NH_4Cl, 0.1 m*M* chloroquine, or 0.1 m*M* monensin or for 5 min with 100 μ*M* phenylarsine oxide for the receptor-mediated endocytosis experiments. Control cells were preincubated for under the same conditions as treated cells but with equivalent dilutions of distilled water substituted for treatments. Unless otherwise indicated all experiments were performed at 37°C and with the addition of 10^4–10^5 cpm/ml ^{32}P-labeled oligonucleotide mixed with unlabeled oligonucleotide to a final concentration of 0.1–10 μ*M*.

Radiolabeled oligonucleotide diluted with unlabeled oligonucleotide was added to cells at $t = 0$. At each sampling time, five 100-μl samples were centrifuged at 1200 rpm for 1 min through 100 μl of 2:7 dionyl phthalate:dibutyl phthalate in a 0.4-ml polypropylene microcentrifuge tube. All samples were then frozen at −70°C. Pellets were clipped off, dissolved in 100 μl of 0.1% sodium dodecyl sulfate by vortexing, then counted in 4 ml of scintillation cocktail (ACSII or Scintiverse II). Counts per minute were converted to cell-associated oligonucleotide based on the known oligonucleotide dilution.

In efflux studies, cells were centrifuged for 20–30 sec after 0.5 to 60 min of incubation with oligonucleotide. The cell pellet was resuspended in an equal volume of fresh medium and processed as described above.

To assess the integrity of the cell membrane after trypsin and azide pretreatments, the following studies were conducted. Approximately 4×10^4 cpm of membrane impermeant [^{14}C]sucrose (6) was added per ml of cells after trypsin or azide pretreatment. Cells were incubated at 37°C for 120 min, then processed as described above. In another test for cell permeability, the cells were incubated at 37°C with 1 μCi of [^{3}H]H_2O/ml of cells for 10 min after trypsin or azide pretreatment. The cells were centrifuged for 1 min, resuspended in fresh medium, and processed as described above.

The stability of the ^{32}P-labeled oligonucleotide was studied by a polyacrylamide gel electrophoresis assay. Oligonucleotide was added to cells at $t = 0$. Aliquots of 250 μl were centrifuged for 30 sec after incubations up to 120 min at 37°C. The extracellular medium was separated from the pellet, and the pellet resuspended in 250 μl of fresh medium. The resuspended pellets and medium were vortexed with 50 μl of 0.1% sodium dodecyl sulfate to lyse the cells. Aliquots containing 2000 cpm were diluted to 40 μl, and 40 μl of 0.025% bromophenol blue in formamide was added to each sample. The samples were heated in boiling water for 3 min, then loaded onto a 19:1 polyacrylamide:bisacrylamide sequencing gel. The samples were run in buffer (0.3 *M* Tris, 0.23 *M* boric acid, 10 m*M* EDTA, pH 8.0) (4) for 2 hr at 600 V. The gel was exposed to X-ray film overnight at −70°C.

Cellular ATP was measured based on a luciferase and luciferin assay using Chrono-Lume Reagent on a ChronoLog Lumivette luminometer according to manufacturer's instructions.

Data are statistically analyzed to determine significance between treatment groups. Analysis of variance allows for evaluation of statistical differences in experiments with greater than two treatment groups. Student's *t* test is a statistical test which compares only two treatment groups at a single time point and is therefore inappropriate for these studies. The analysis of variance is calculated using STATWORKS (Version 1.2, Cricket Software, Philadelphia, PA). The reported *P* values represent the probability of making an error in determining differences between treatment groups.

A kinetic modeling program, STELLA (version 2.1), was used to describe and simulate the experimental data. Kinetic constants were chosen to result in a reasonable simulation of the data.

RESULTS

In general, oligonucleotide uptake curves show rapid cell association in the first minute, followed by a slower uptake process. Oligonucleotide uptake then appears to approach steady-state conditions. Less than 2% of the total added oligonucleotide becomes cell associated after a 120-min incubation at 37°C, resulting in cell-associated concentrations one-third to one-fifth of the extracellular oligonucleotide concentration.

We find that a component of the uptake of oligonucleotides into Rauscher cells requires energy. Inhibition of cellular energy production by reduced temperature or by the metabolic inhibitor azide results in a significant, but incomplete reduction in oligonucleotide uptake ($P < 0.05$) (Figs. 1 and 2). Pretreatment with another metabolic inhibitor, 2-deoxyglucose, also significantly reduces uptake ($P = 0.01$). Azide pretreatment reduces cellular ATP to 60% of controls.

A trypsin-sensitive component is also found to be important in cell association of this oligonucleotide (Fig. 2). Trypsin pretreatment partially reduces rapid binding seen within the first minute and uptake after the first minute. Simultaneous administration of azide and trypsin does not further reduce cell association below trypsin treated levels, implying that the trypsin-sensitive component may be responsible for internalization and is the energy-utilizing step in oligonucleotide uptake. Degradation of the oligonucle-

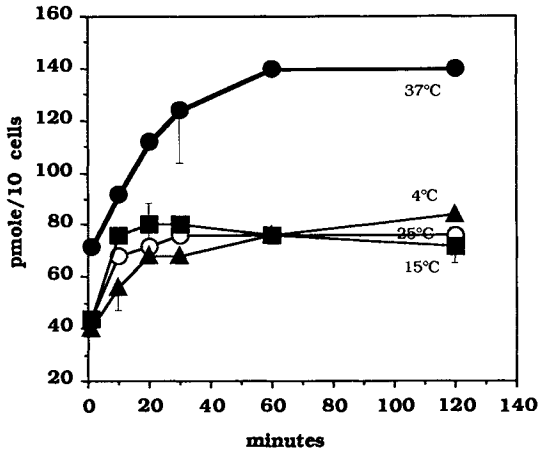

Fig. 1. Cells are preequilibrated at 4, 15, 25, or 37°C. At $t = 0$, oligonucleotide is added to the cells such that the final concentration is 0.1 μ*M*. Samples are taken between 1 and 120 min. Each point represents the mean of three experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

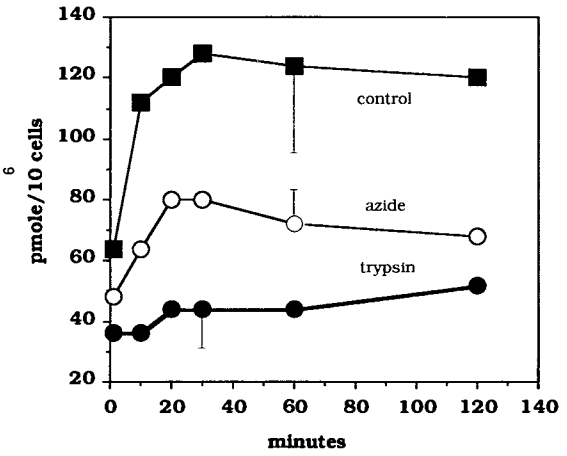

Fig. 2. Cells are preincubated with 0.25% trypsin or 10 m*M* azide for 60 min at 37°C. At $t = 0$, oligonucleotide is added to the cells such that the final concentration is 0.1 μ*M*. Samples are taken between 1 and 120 min. Each point represents the mean of six experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

otide due to trypsin is not observed during a 2-hr incubation at 37°C. Neither uptake of the fluid-phase marker [^{14}C]sucrose (control, 25 ± 6 cpm; azide, 25 ± 3 cpm; trypsin, 28 ± 12 cpm) nor leakage of [^{3}H]H_2O (control, 51 ± 28 cpm; azide, 60 ± 5 cpm; trypsin, 85 ± 6 cpm) in azide- or trypsin-treated cells is found to differ from untreated controls.

The amount of extracellularly bound oligonucleotide is also studied. After an hour of exposure to labeled oligonucleotide, cells are diluted in medium immediately prior to centrifugation with or without a 100-fold excess of unlabeled oligonucleotide. These dilutions remove approximately 10–30% of the oligonucleotide associated with the cell pellet. No difference is seen between dilutions containing and dilutions lacking unlabeled oligonucleotide. Pretreatment of the cells with trypsin does not affect the amount of oligonucleotide removed by dilution. When the cells are resuspended in fresh medium instead of diluted, a rapid dissociation occurs in the first minute, followed by stabilization of cell-associated oligonucleotide. This rapid dissociation results in loss of approximately 40–50% of cell-associated oligonucleotide. Next, in order to differentiate nonspecific interactions from trypsin-sensitive surface binding, oligonucleotide-loaded cells are treated with 1.7% trypsin at 37°C. This treatment removes approximately 30% of cell-associated oligonucleotide within the first minute and subsequent resuspension removes an additional 30%, leaving approximately 40% of cell-associated oligonucleotide remaining after resuspension and trypsin treatment. Cells which are incubated with labeled oligonucleotide for only 30–60 sec, then resuspended in trypsin-containing medium, result in almost-complete removal of cell-associated oligonucleotide (<5 pmol/10^6 cells). In addition, the antisense 21-mer complementary to the c-*myc* protooncogene, which is involved in cellular proliferation, at a 0.1 μ*M* final concentration is found to inhibit cellular proliferation significantly. Cellular proliferation is inhibited by 44% compared to controls by 3 days ($P < 0.05$). These results suggest that up to 40% of cell-associated oligonucleotide becomes internalized, whereas up to 60% of uptake is due to a combination of trypsin-sensitive and trypsin-insensitive surface binding.

To assess the effect of charged compounds on oligonucleotide cell association, a 100-fold molar excess of ATP, random-sequence oligonucleotide, salmon sperm DNA, or dextran sulfate is preincubated with the cells. This preincubation is found to result in a significant reduction in the extent of uptake in all four cases compared to the control ($P < 0.05$) (Fig. 3). Unlabeled antisense c-*myc* oligonucleotide also competes with ^{32}P-labeled oligonucleotide (Fig. 4). Uptake is found to be concentration dependent ($P < 0.01$) and not saturated up to 10 μ*M* (Fig. 5).

Pretreatment of cells with the anion channel inhibitor SITS results in a significant reduction in oligonucleotide uptake ($P < 0.05$) (Fig. 6), as does preincubation with excess chloride and sulfate ($P < 0.05$) (Fig. 7). The addition of 10 m*M* NH_4Cl, NaCl, or Na_2SO_4 results in a final chloride concentration of 134 m*M* or a sulfate concentration of 10.8 m*M* in minimal essential medium-alpha. When the cells are pretreated with inhibitors of receptor-mediated endocytosis, the inhibitors monensin and chloroquine are shown to have no effect on uptake, whereas NH_4Cl significantly decreases oligonucleotide cell association (Fig. 8). No significant difference is found at time = 1 min among the four treatment groups as measured by *t* test. Phenylarsine oxide also fails to inhibit oligonucleotide uptake ($P < 0.05$).

Gel electrophoresis demonstrates no significant degradation of the oligonucleotide in the cells after a 120-min incubation or in the extracellular medium after a 60-min incubation. Between 60 and 120 min, 28% of the oligonucleotide in the medium has been degraded into a smaller oligonucleotide, monomer, or free phosphate (Fig. 9). Free phosphate and [^{32}P]ATP are found to migrate the same distance in the gel. Therefore, more than 50% of the oligonucleotide remains intact in the medium for the duration of this experi-

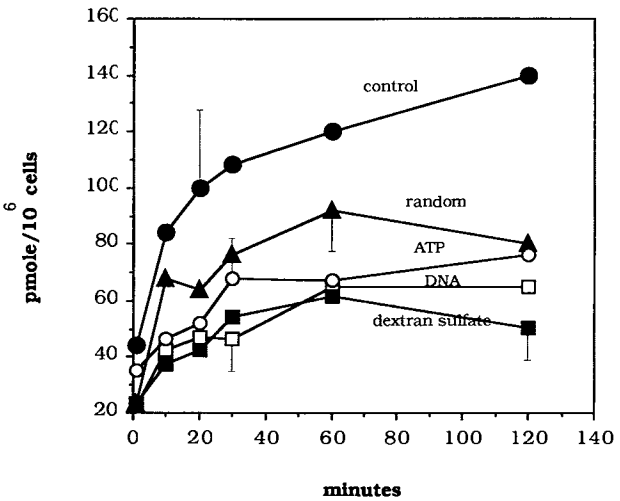

Fig. 3. Cells are preincubated with 10 μ*M* salmon sperm DNA, random oligonucleotide, ATP, or dextran sulfate for 1–4 min at 37°C. At $t = 0$, oligonucleotide is added to the cells such that the final concentration is 0.1 μ*M*. Samples are taken between 1 and 120 min. Each point represents the mean of six experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

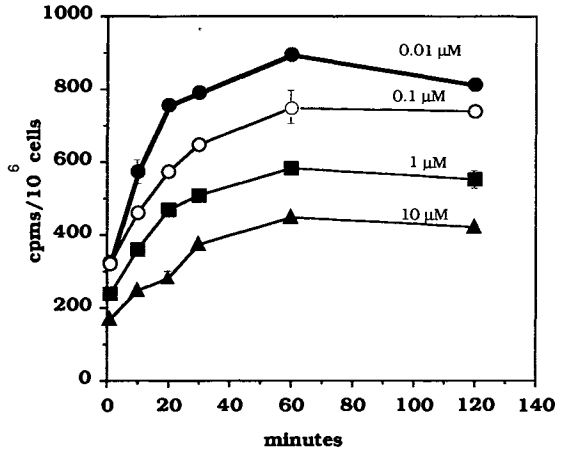

Fig. 4. Approximately 10^6 cpm of ^{32}P-labeled oligonucleotide is diluted with unlabeled oligonucleotide and is added to the cells at t = 0 such that the final oligonucleotide concentration added is 0.01, 0.1, 1.0, or 10 μM. Samples are taken between 1 and 120 min. Data are from a representative experiment. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SD.

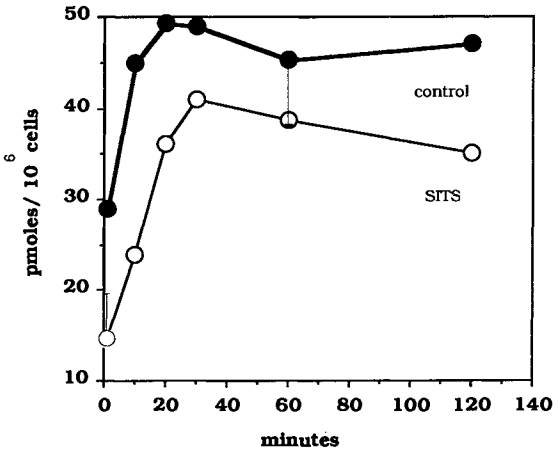

Fig. 6. Cells are preincubated with 60 μM SITS for 60 min at 37°C. At t = 0, oligonucleotide is added to the cells such that the final concentration is 0.1 μM. Samples are taken between 1 and 120 min. Each point represents the mean of three experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

ment and oligonucleotide degradation within the cell is not significant in the time period studied.

DISCUSSION

Antisense oligonucleotides have been used experimentally for more than a decade as specific inhibitors of target gene expression (1,7). The oligonucleotides are designed to base-pair specifically by Watson–Crick hydrogen-bonding to complementary RNA. The target RNA may be either heteronuclear RNA normally found in the cell nucleus or mRNA found in the cytoplasm (8–11). The resulting hybrid is thought to prevent RNA processing by interfering with splice protein or ribosome binding. An enzyme that degrades the RNA in a RNA–DNA duplex, RNAse H, also plays a role in antisense oligonucleotide inhibition of gene expression (12,13).

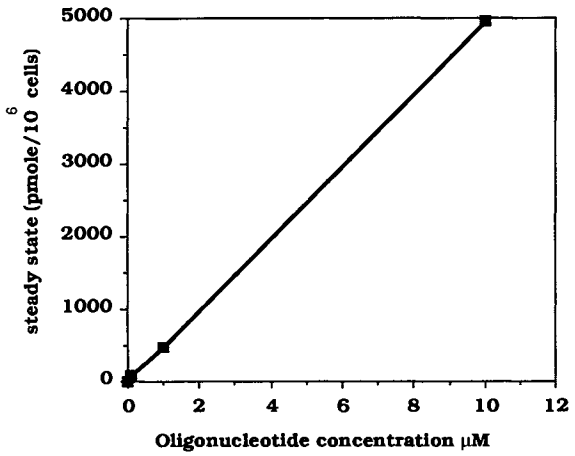

Fig. 5. At t = 0, oligonucleotide is added to the cells such that the final concentration is 0.01, 0.1, 1, or 10 μM. The steady-state value of uptake is plotted versus the added oligonucleotide concentration. Data are from a representative experiment.

Gene expression can be selectively inhibited either by adding the antisense oligonucleotide to the cell-containing medium, by introducing the oligonucleotide directly into the cytoplasm by microinjection (14), or by using a plasmid vector which expresses an antisense RNA sequence inside the cell (15,16). It is assumed that the oligonucleotide is able to reach the site of action while simultaneously maintaining sequence specificity. The highly negatively charged oligonucleotides are unlikely to cross the cell membrane by simple diffusion. Other traditional routes of molecular uptake in mammalian cells normally result in macromolecular degradation, such as endocytosis, or are traditionally used for much smaller molecules as in carriers or cotransporters. Therefore, because no known cell membrane transporter can account for the uptake and activity of antisense oligonucleotides, their mechanism of uptake may prove to be unique.

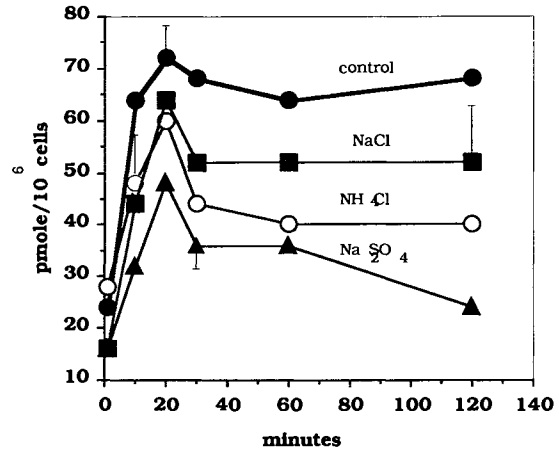

Fig. 7. Cells are preincubated with 10 mM NH_4Cl, NaCl, or Na_2SO_4 for 15 min at 37°C. At t = 0, oligonucleotide is added to the cells such that the final concentration is 0.1 μM. Samples are taken between 1 and 120 min. Each point represents the mean of four experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

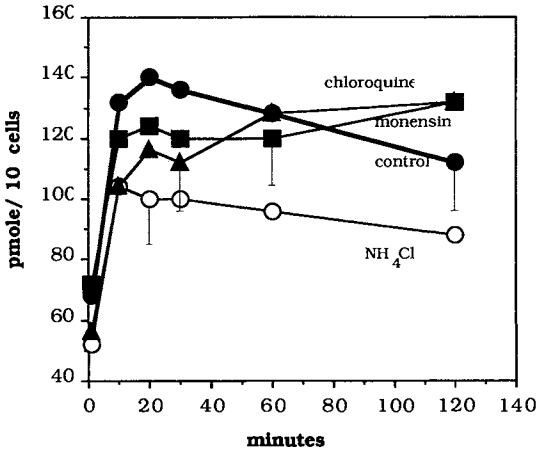

Fig. 8. Cells are preincubated with 10 m*M* NH_4Cl, 0.1 m*M* chloroquine, or 0.1 m*M* monensin for 30 min at 37°C. At $t = 0$, oligonucleotide is added to the cells such that the final concentration is 0.1 μ*M*. Samples are taken between 1 and 120 min. Each point represents the mean of five experiments. As a visual aid, typical errors at selected values are represented by vertical bars that are 1 SE.

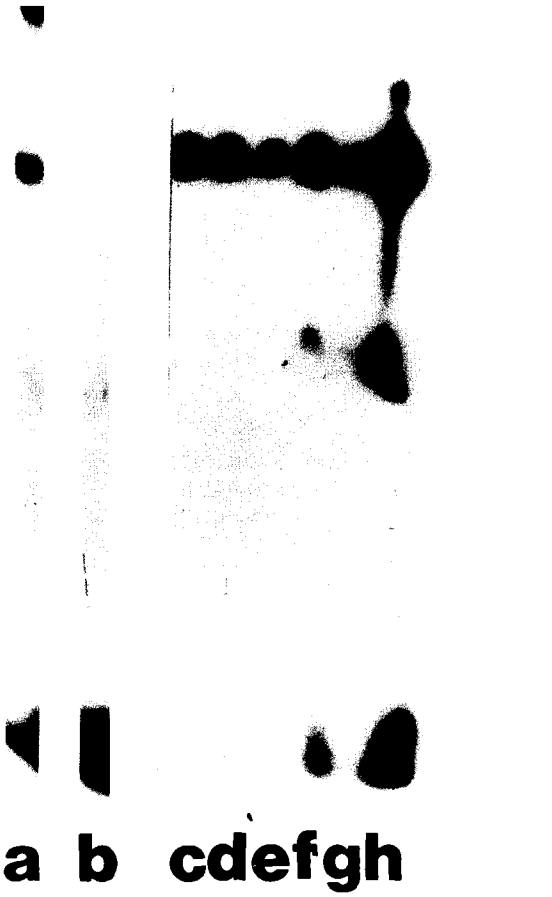

Fig. 9. Extracellular medium and cells are electrophoresed after incubation with ^{32}P-labeled oligonucleotide for 1 to 120 min. Approximately 2000 cpm is loaded onto a 19% polyacrylamide sequencing gel and electrophoresed for 2 hr. The controls are (a) ^{32}P-labeled oligonucleotide and (b) free ^{32}P. The ^{32}P-labeled oligonucleotide obtained from the cells appears in lanes c, e, and g, and that from medium in lanes d, f, and h. The cells are incubated with the ^{32}P-labeled oligonucleotide, then separated from the medium at 1 min (lanes c and d, 60 min (lanes e and f, and 120 min (lanes g and h).

Evidence of protein involvement in the cellular uptake of oligonucleotides exists, but the nature of the mechanism remains unknown (3,17). Loke *et al.* (3) have identified a putative 80-kd receptor protein with a high affinity for an oligothymidylate. The oligothymidylate labeled with a 5′-acridine exhibits a punctate intracellular pattern when incubated with the HL-60 cells. The authors conclude that oligonucleotide uptake occurs via a receptor-mediated endocytic pathway. The punctate fluorescence of an acridine-labeled oligonucleotide is confirmed by Stein *et al.* (18). If this punctate pattern is caused by oligonucleotides inside lysosomal or prelysosomal vesicles, then it remains unclear how the oligonucleotide is able to survive lysosomal enzymes and escape to the site of action.

In this study, we address the mechanism of oligonucleotide uptake using a 21-base single-stranded deoxyribonucleic acid oligonucleotide (21-mer) complementary to the translation initiation region of the c-*myc* proto-oncogene. The antisense c-*myc* 21-mer is chosen because similar oligonucleotides have been shown previously to have biological activity (19–21). Our studies demonstrate that the antisense c-*myc* oligonucleotide inhibits cellular proliferation. The sequence of the oligonucleotide is not expected to play a role in the uptake process, although oligonucleotide length and composition may influence uptake. We are not aware of any evidence for sequence specificity in cellular uptake of oligonucleotides.

The sequence specificity of oligonucleotides is based on the presumption that the oligonucleotide binds to the target RNA in the nucleus or cytoplasm. This hypothesis suggests that the site of action for an intact oligonucleotide is an intracellular location. Preliminary studies using an antisense c-*myc* 21-mer labeled with fluorescein isothiocyanate show intracellular fluorescence when incubated with the cells, an observation not seen with free fluorescein isothiocyanate (data not shown). The fluorescence studies and proliferation assays provide qualitative evidence that a portion of the antisense oligonucleotide used in this study is internalized by the cell. In addition, efflux studies and washes with and without trypsin result in incomplete removal of cell-associated oligonucleotide. These studies demonstrate that, to some extent, the c-*myc* antisense oligonucleotide associates with the cell surface and is internalized.

Because oligonucleotide uptake is partially reduced as a result of metabolic inhibitor pretreatment or exposure to low temperature, a portion of association involves cellular energy (Figs. 1 and 2). This energy requirement may be due directly to a primary active transport process or indirectly to other processes such as secondary active transport or maintenance of cell membrane potential, structure, or integrity. Failure to reduce uptake completely in these studies may be due partially to incomplete ATP depletion by azide or may represent a large surface binding contribution in the absence of internalization. However, the extent of uptake at low temperatures after 120 min coincides with first-minute binding at 37°C and is therefore consistent with cell surface binding.

The reduction in oligonucleotide uptake after trypsin

pretreatment suggests that a cell surface protein, possibly a receptor, is necessary for oligonucleotide uptake (Fig. 2). Following treatment with 0.25% trypsin, cell association occurs only in the first minute. The magnitude of this rapid binding is comparable to the low-temperature surface association described above and so may be due to nonspecific, trypsin-insensitive surface binding or incomplete trypsinization. The absence of continued uptake after initial rapid binding implies that oligonucleotide internalization occurs via a trypsin-sensitive component. The observed inhibition of uptake due to trypsinization may also be due to an indirect effect, such as perturbation of the properties of the lipid bilayer. We conclude from these studies that uptake consists of a trypsin-sensitive and a trypsin-insensitive component.

The oligonucleotide bound to the cell surface likely accounts for the rapid efflux seen in the efflux and wash studies. Following rapid efflux, cell-associated oligonucleotide stabilizes, suggesting that the oligonucleotide is undergoing neither secretion nor transcytosis. Wash studies demonstrate that approximately 60% of cell-associated oligonucleotide is surface bound after a 60-min incubation with oligonucleotide. Half the surface-bound oligonucleotide is removed by trypsinization, and the other half is removed by resuspension. Preloading cells with oligonucleotide for 30–60 sec, followed by resuspension and trypsinization, results in almost-complete removal of cell-associated oligonucleotide. These results are consistent with the hypothesis that cell surface binding occurs rapidly and consists of a trypsin-sensitive and a trypsin-insensitive component.

The addition of charged species is found to reduce oligonucleotide uptake nonspecifically. Oligonucleotide uptake is decreased by preincubating the cells with nucleic acids, a nonnucleic acid polyanion, and dextran sulfate (Figs. 3 and 4). The findings by Loke *et al.*, that a 5′ phosphate is necessary for oligonucleotide uptake inhibition, are therefore inconsistent with these results (3). We find that SITS (Fig. 6), chloride, and sulfate (Figs. 7 and 8) also reduce oligonucleotide uptake. At a 60 μ*M* final concentration, SITS inhibits red blood-cell anion channels which transport small anions such as chloride or sulfate (22). However, because only small anions are known to traverse anion channels, nonspecific interaction is the most probable explanation. Channels which transport macromolecules such as DNA have not yet been described in mammalian cell membranes, although evidence for such transporters has been demonstrated in mitochondria (23,24). In nonmammalian cells, channels are used by bacterium in genetic transformation. A T4 phage virus is known to carry a channel which is used to inject its DNA into its host *E. coli* (25). Whether the reduction in uptake is due to inhibition of the anion channel or due to nonspecific charge–charge interactions has yet to be determined.

The effects of inhibitors of receptor-mediated endocytosis are also tested to examine the role of receptor-mediated endocytosis in oligonucleotide uptake. Endosomal acidification is required in some systems for receptor recycling (26), endosome/lysosome fusion (27), and full activity of lysosomal enzymes. Monensin, chloroquine, and NH_4Cl prevent the acidification of endosomes and lysosomes. In this study, monensin and chloroquine have no effect on uptake, whereas NH_4Cl significantly decreases oligonucleotide cell association (Fig. 8). Why NH_4Cl demonstrates an inhibitory effect whereas monensin and chloroquine do not is unclear, but the effect may be an example of the charge effect discussed above. These results are also inconsistent with the findings of Loke *et al.* (3), who demonstrate that chloroquine causes accumulation of a 5′-acridine-labeled oligothymidylate. This inconsistency may be due to the 5′ modification of their oligothymidylate, the different composition of the oligonucleotide, or the cell lines used. Phenylarsine oxide, an endocytosis inhibitor of compounds known to be endocytosed via a receptor (28,29), also fails to inhibit oligonucleotide uptake in these studies. These results in combination with those discussed above diminish the probability of receptor-mediated endocytosis being the primary route of oligonucleotide centry into these cells.

Clearly, oligonucleotide uptake is a complex process even under serum-free conditions. The observations presented here are consistent with two intracellular, one extracellular, and two surface components as depicted in Fig. 10a. The model is useful in that it schematically summarizes our data. However, because of the complexity of oligonucleotide interactions with cells, there may be other schemes that account equally well for the observations presented. We hypothesize that surface binding of unmodified oligonucleotides consists of a slowly equilibrating (i.e., trypsin-sensitive) and a rapidly equilibrating (i.e., trypsin-insensitive) component, the former resulting in cellular internalization. The first intracellular component (intracellular 1) is proposed to equilibrate with the trypsin-sensitive surface component. The second (intracellular 2) may represent saturable, high-affinity binding within the cell and accounts for cellular retention of the oligonucleotide. The extracellular concentration is maintained constant throughout the simulation. Equilibrium constants are chosen in order to simulate oligonucleotide uptake and cell association in the absence of inhibitors.

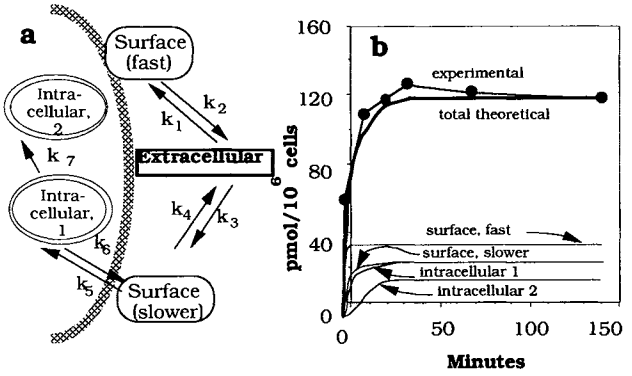

Fig. 10. (a) Summary of essential observations is presented as a kinetic model consisting of one extracellular component, one surface component with rapid association (k_1 = 30 min^{-1}) and dissociation (k_2 = 300 min^{-1}), and one surface component with slow association (k_3 = 0.075 min^{-1}) and dissociation (k_4 = 1 min^{-1}). Internalization occurs from the slow-binding component (k_5 = 0.75 min^{-1}) (intracellular 1), followed by efflux (k_6 = 0.75 min^{-1}) or transfer to a saturable compartment (intracellular 2) (k_7 = 0.05 min^{-1}). Rate constants are chosen to match experimental observations reasonably. (b) The simulated oligonucleotide concentration associated with each component (amount/cell volume) is shown over time.

Uptake in the presence of inhibitors is simulated by reducing the binding (k_3) or uptake (k_5) rate constants 100-fold. These models also result in a reasonable simulation of the experimental data. Because the experimental data suggest that internalization occurs from the slowly equilibrating, trypsin sensitive component, models in which internalization occurs from the fast-equilibrating, trypsin-insensitive component are not presented in this paper. Models with fewer than five compartments or seven rate constants are found to result in inferior data simulation. For example, if k_6 is removed to simulate an active, one-way transport process into the cell, the curves simulated with azide pretreatment or the efflux model result in inferior fits compared to those when k_6 is present. Although we believe that this model is the simplest model capable of simulating our data, other more complex models are likely to also result in reasonable simulations. This model also does not account for the complications resulting from the addition of serum to the extracellular compartment.

The data in this study are consistent with oligonucleotide uptake occurring via a complex process which includes a trypsin-sensitive component and cellular energy. The absence of significant inhibition of uptake by receptor-mediated endocytosis inhibitors implies that uptake does not occur via a traditional receptor-mediated endocytosis mechanism in these cells. Whether or not a channel of this nature is involved in oligonucleotide internalization, the physiological significance of the oligonucleotide transport system, its distribution across cell lines, the mechanism of transport, and methods to optimize oligonucleotide uptake are areas which merit further study.

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