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Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages

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ABSTRACT

The cellular uptake and intracellular distribution of methylphosphonate oligonucleotides (15 mers) has been examined using both ^{32}P labeled and fluorescent labeled oligonucleotides. The cellular uptake process for methylphosphonate oligonucleotides is highly temperature dependent, with a major increase in uptake occuring between 15 and 20°C. Most of the label which becomes cell associated at 37°C cannot be removed by acid washing or trypsinization and thus seems to be within the cell. Visualization of rhodamine labeled methylphosphonate oligonucleotides using digital imaging fluorescence microscopy reveals a vesicular subcellular distribution suggestive of an endosomal localization. There was extensive co-localization of rhodamine labeled methylphosphonate oligonucleotides with fluorescein-dextran, an endosomal/lysosomal marker substance. The apparent endocytotic uptake of labeled methylphosphonate oligonucleotides could not be blocked by competition with unlabeled methylphosphonate or phosphodiester oligonucleotides, nor by ATP. This contrasts with the situation for radiolabeled phosphodiester oligonucleotides whose uptake can be completely blocked with unlabeled competitor. Uptake of phosphodiester oligonucleotides, but not of methylphosphonate oligonucleotides, could be blocked by acidification of the cytosol. These observations suggest that the pathway of cellular uptake of methylphosphonate oligonucleotides involves fluid phase or adsorbtive endocytosis, and is distinct from the uptake pathway for phosphodiester oligonucleotides.

INTRODUCTION

Antisense oligonucleotides seem to have considerable promise as therapeutic agents for viral diseases and cancer [1–4]. Antisense compounds can potentially interdict the expression of specific genes by interfering with transcription, message processing, RNA stability, or translation [5]. In order to exert any of these effects, the oligonucleotides must enter into the cytoplasmic and nuclear compartments of the cell. From the perspective of many years of study of membrane transport phenomena, it is quite remarkable that oligonucleotides enter cells at all, since other compounds of smaller size and lower charge are usually thought to be unable to cross the cell membrane [6,7]. Nonetheless, it is clear that oligonucleotides do enter cells in quantities sufficient to produce impressive biological effects in several different systems [1–5]. Investigations on the mechanism of cellular uptake of unmodified phosphodiester-linked oligonucleotides have indicated that endocytosis seems to be the major pathway, and that there are specific cell surface binding proteins for oligodeoxynucleotides of about 65–80kD which may play a role in uptake [8,9]. The uptake process for phosphodiester oligodeoxynucleotides is temperature and energy dependent and is inhibitable by excess unlabeled oligodeoxynucleotide [8].

Since unmodified phosphodiester oligodeoxynucleotides are readily degraded by nucleases present in cells and in extracellular fluids [10,31], there has been a considerable effort devoted to synthesis of chemically modified oligodeoxynucleotides which are nuclease resistant while still retaining Watson-Crick base pairing specificity. While many different types of chemically modified oligodeoxynucleotides have been synthesized [11], the most widely used modifications are the replacement of a backbone oxygen with sulfur to form a phosphorothioate [3,12], or the substitution of a methyl residue to form a methylphosphonate [4,11,13]. Phosphorothioate oligodeoxynucleotides retain the anionic charge characteristics of phosphodiester oligodeoxynucleotides, while methylphosphonate oligodeoxynucleotides are uncharged. Little is known about the cellular uptake characteristics of modified oligodeoxynucleotides. Phosphorothioates have been reported to competively block the cellular uptake of phosphodiester oligodeoxynucleotides and thus may enter cells by the same mechanisms [12,14]. By contrast, methylphosphonate oligodeoxynucleotides do not compete with charged oligodeoxynucleotides for uptake [8]. Because of their relatively poor aqueous solubility and their lack of charge, it has been suggested that methylphosphonate oligodeoxynucleotides enter cells by

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passive diffusion across the cell membrane [13,14]; however, studies to date have been rather preliminary and have not clearly delineated an uptake mechanism.

In this report we examine the cellular uptake and intracellular distribution of oligonucleotides containing methylphosphonate linkages. We synthesized 15-mer methylphosphonate oligodeoxynucleotides complementary to human *mdr*1 mRNA, and investigated their uptake by a drug resistant Chinese Hamster Ovary cell line (CH^RC5) [15]. The oligonucleotide used here for cell uptake studies is similar to ones currently being employed in our laboraratory in functional studies [Akhtar & Juliano, in preparation]. Since the cellular uptake of a labeled compound may be affected by the chemical characteristics of the label, we have used two different types of labeled methylphosphonate oligonucleotides. One was labeled at the 5′ end with ^{32}P, while the other was labeled with rhodamine covalently attached to a 5′ amino linker group. The rhodamine labeled oligodeoxynucleotides were also employed for studies of intracellular distribution using digitized fluorescence microscopy. Our results suggest that oligodeoxynucleotides containing methylphosphonate linkages are taken up by cells through a process of active endocytosis, but that this process is distinct from that responsible for uptake of standard phosphodiester oligodeoxynucleotides.

MATERIALS AND METHODS

Synthesis of oligodeoxynucleotides

Methylphosphonate containing oligodeoxynucleotides (MP-oligonucleotides, 15mers) with a sequence complementary to a site overlapping the AUG initiation codon of the human *mdr*1 mRNA [15] were synthesized on an automated DNA synthesizer (Model 380B, Applied Biosystems, Foster City, CA) using beta-cyanoethyl cycles [16,17]. Methyl phosphonamidate synthons were obtained from Applied Bionetics, Hayward, CA. The sequence was 5′ CTC CAT CAC CAC CTC 3′ and contained a single 5′ end phosphodiester moiety to facilitate radiolabeling with T4 kinase. MP-oligonucleotides with an amino linker group were also synthesized by attaching an amino hexyl phosphate linker (Aminolink 2; Applied Biosystems, Foster City, CA) to the 5′ end of the oligodeoxynucleotide in the last coupling cycle. The oligodeoxynucleotides were detritylated, deprotected and purified according to a standard protocol [16].

5′ end labelling of oligodeoxynucleotides with [^{32}P]

MP-oligonucleotides were 5′ end labeled with [^{32}P]-gamma-labelled ATP using T4 polynucleotide kinase (Promega) in buffer containing 100 mM Tris pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA at 37°C for 30 minutes. Specific radioactivity was 10^8-10^9 cpm/μg. Labelled oligodeoxynucleotides were purified by denaturing PAGE (15% polyacrylamide containing 7 M urea). The band corresponding to the desired oligodeoxynucleotide was eluted with 20% ethanol/water, desalted by ethanol precipitation, or by gel filtration on Sephadex G-25 (DNA grade superfine, Pharmacia, Sweden), resuspended in isotonic phosphate buffered saline, and stored at −20°C until use.

Rhodamine labeled oligodeoxynucleotide

MP-oligonucleotides with an amino linker (50μg) were incubated with 100μg tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC, purchased from Research Organics Inc, Cleveland, OH) in sodium carbonate/sodium hydrogen carbonate buffer (pH 9.0) at room temperature overnight. The labeled oligodeoxynucleotide was purified by Sephadex G-25 (DNA grade) column chromatography to remove free TRITC. The purity of the conjugate and the absence of free TRITC was checked by PAGE.

Transport studies

Cellular uptake studies were carried out using CH^RC5 cells (a multiple drug resistant mutant of the Chinese Hamster Ovary tumor cell line) [15,18]. These cells were kindly provided by Dr. V.Ling (Ontario Cancer Institute, Toronto, Ontario, Canada). The cells were routinely maintained by serial culture in alpha-MEM supplemented with 10% v/v FCS and 1% antibiotics at 37°C in a 5% CO_2 atmosphere. Under these conditions the cells were exponentially growing with a doubling time of about 18 hrs. This cell line was found to be free of Mycoplasma.

For uptake experiments, $1-2\times10^5$ cells/well (24 well plate) were incubated with 5′-[^{32}P] labelled MP-oligonucleotides in alpha-MEM medium containing 10% v/v heat inactivated FCS; in some experiments serum-free HEPES buffer containing 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid), 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 1% bovine albumin (pH 7.4) was used instead. When inhibitors were used, these were added 15 min. prior to addition of labeled MP-oligonucleotides. After the incubation, the medium was removed and cells washed 4 times with 0.5 ml PBS. Cells were then lysed with 1% SDS and cell associated radioactivity was determined by liquid scintillation counting. Cells were incubated over a range of 4°C−37°C for the studies on temperature dependency of uptake; all other uptake studies were carried out at 37°C.

To strip off surface bound oligodeoxynucleotides, 0.4 M acetate buffer (pH 2.3) containing 1M NaCl was used as a washing solution. Low pH washing has been reported to remove almost 90% of cell surface bound ligands[19]. Alternatively, trypsin-EDTA was used to remove membrane associated oligodeoxynucleotides by digesting cell surface protein binding sites. Cells were treated with 200μl of trypsin-EDTA(×1) (Sigma) until all cells became round and came off the plates; 1ml of PBS was added, and the cells and were recovered by centrifugation at 1000 rpm for 5min. Cells were washed with PBS and lysed with 1% SDS. Cell associated radioactivity was determined by liquid scintillation counting.

Digital video imaging system

Experiments on the subcellular distribution of oligonucleotides were performed using the MDVM (multiparameter digitized video microscopy) imaging system described elsewhere [20,21]. The MDVM system basically consists of an epi-fluorescence microscope (IM-35; Carl Zeiss, Inc.), excitation and neutral density filter wheels, video camera and a digitizing board interfaced to a computer. The excitation wavelength was provided by a 100-W mercury vapor lamp and passed through computer controlled interference and neutral density filter wheels. A band pass excitation filter of 545 nm, a long pass barrier filter of 590nm, and a dichromatic beam splitter of 580nm were used to visualize the subcellular distribution of TRITC labeled MP-oligonucleotides. The microscope stage was maintained at 37°C by an air curtain incubator. The fluorescent image of the cell

was projected by a rifle-telescope attached to the microscope on to the computer controlled image intensifier Gen11sys Camera. The Gen11sys consists of a head, including proximity type MCP (microchannel plate) image intensifier with relay optics, coupled to a high gain CCD-72 (charged coupled device) camera (model Gen11sys, Dage-MIT, Inc., Michigan City, IN). Both the Gen11sys and CCD-72 camera has their own control units to manipulate and fix the gain and blacklevel of the image during the experiments. The resultant image from the CCD camera was digitized by the imaging board (512×512 pixel, 8bit, model IP512, Imaging Technology, MA), and interfaced to a PDP 11/23 Computer (Digital Equipment Corp., Maynard, MA). A 63× oil immersion plan-Neofluar objective (NA=1.25) or 40× oil immersion Nikon-Fluor was used to observe the fluorescent image, and 1.5−3.0× was used at the rifle telescope. During the experiments, the live cell fluorescence was averaged over 256 images or 32 images, background subtracted, and stored to the disk as a 256×256 pixel image. This stored fluorescent image was used to analyze the data. To measure the fluorescence intensities, 50 different fields were recorded. Phase contrast images were also recorded.

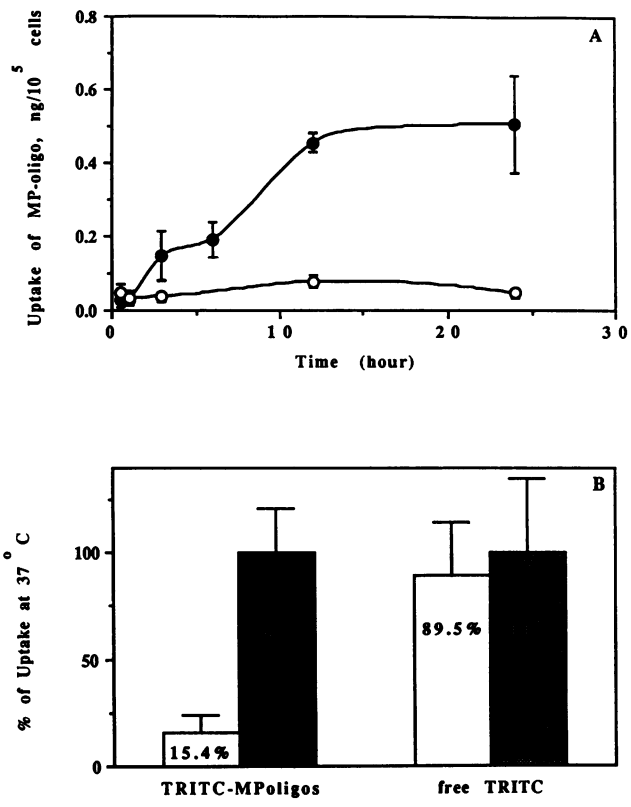

Fig. 1. Effects of temperature and time on cellular uptake of methylphosphonate oligodeoxynucleotides. (A) CH^RC5 cells were incubated with 0.01μM [^{32}P] labelled MP-oligodeoxynucleotides at 4°C (open) or 37°C (closed) in 10% heat inactivated FCS containing alpha-MEM for various times. Amounts of cell associated MP-oligonucleotides are expressed as ng/10^5 cells. Each experiment is in quadruplicate. (B) Cells were incubated 4μM TRITC labeled MP-oligodeoxynucleotides or free TRITC in HEPES buffer at 4°C (clear) or 37°C (solid) for 1 hour. Amount of fluorescence intensity was determined by digitized video imaging system as described in 'Materials and Methods'. Fluorescence intensities were averaged for 50 fields. Each field contained 10−20 cells. Uptake at 4°C was determined as percentage of fluorescence at 37°C in each case. Bars are standard deviations.

Cell culture for digital video imaging

For experiments concerning the subcellular distribution of MP-oligonucleotides, cells were cultured on glass coverslips in alpha-MEM containing 10% FCS for 2 or 3 days. Thereafter 4uM TRITC labelled MP-oligonucleotides were incubated with cells in HEPES buffer (pH 7.4) at 37°C in 5% CO_2. With fluorescent oligonucleotides, uptake studies in HEPES buffer rather than serum containing growth medium provided a higher level of cell uptake and lower background fluorescence(data not shown); thus HEPES buffer was used for the experiments on subcellular distribution in order to get sufficient fluorescence intensity. To identify endocytic/lysosomal vesicles, cells were pre-incubated with 2 mg/ml FITC dextran (Molecular Probes, OR), a well known marker for the endosome-lysosome compartment [22], in complete alpha-MEM medium at 37°C for 1 hour. Then cells were washed with HEPES buffer and were incubated with 2mg/ml of nonlabeled dextran in a complete alpha-MEM medium at 37°C. After overnight incubation, cells were washed with HEPES buffer, and 4uM TRITC labeled MP-oligonucleotides were added in HEPES buffer. After 1 hour incubation, coverslips were washed throughly with HEPES buffer containing 0.1% BSA and mounted in a special chamber designed for the digital video imaging system. During fluorescence microscopy, HEPES buffer was used to prevent shifts in extracellular pH which might change the sub-cellular distribution patterns.

Inhibition of receptor mediated endocytosis

It has been reported that reduction of cytoplasmic pH inhibits receptor-mediated endocytosis but not fluid-phase endocytosis [23,24] . Thus the transport mechanism of MP-oligonucleotides was examined under conditions of low cytoplasmic pH. For the acidification of intracellular pH (pHi), extracellular pH (pHo) was controlled with bicarbonate-free alpha MEM supplemented with 0.2% BSA and buffered with 20 mM MES (2,(N-morpholino)-ethanesulfonic acid, Sigma). In each experimental situation, pHi was measured in cells loaded with BCECF-AM (2′,7′-bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester, Molecular Probes) using the digitized video image system, as described elsewhere [25].

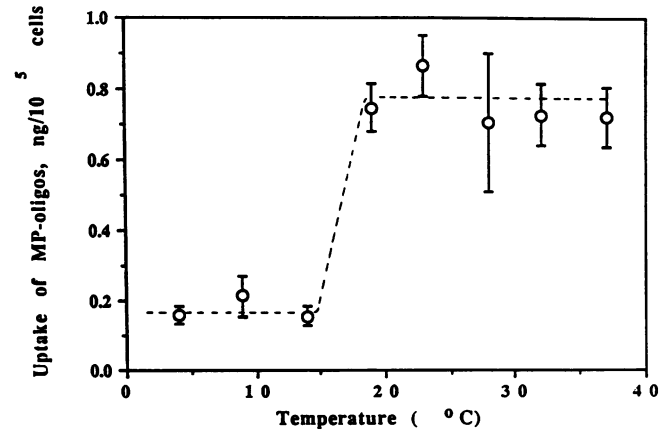

Fig. 2. Temperature dependence of cellular uptake of methylphosphonate oligodeoxynucleotides. CH^RC5 cells were incubated with 0.01μM MP-oligodeoxynucleotides in HEPES-buffer (pH7.4) at 4, 9, 14, 19, 23, 28, 32, 37°C for 3 hours. Cell-associated radioactivity was determined by liquid scintillation counting. Uptake is expressed as ng/10^5 cells. Bars are standard errors. Experiments were carried out in triplicate. Bars are standard deviations.

Intracellular stability of MP-oligonucleotides

Cells were incubated in alpha-MEM with 10% v/v heat inactivated FCS containing 5'-[32P] labeled MP-oligonucleotides or D-oligonucleotides in a 24 well plate at 37°C for various times. The incubation mixtures were removed and the cells were scraped out using a rubber policeman. Cell pellets were spun down and washed with PBS. Cells were lysed by 0.5% NP-40/PBS and were heated at 65°C for 30 min. Incubation supernatants and the original MP-oligonucleotides or D-oligonucleotides were also treated in the same manner. Samples were analyzed by PAGE (15% denaturing gel containing 7M urea).

RESULTS

Temperature and time dependence of cellular uptake of MP-oligonucleotides

Figure 1A shows the effects of temperature and time on cellular uptake of 5'-[^{32}P] labeled MP-oligonucleotides in CH^{R}C5 cells. Significant uptake of MP-oligonucleotides was observed at 37°C and plateaued at 10−12 hours. The major difference between cellular uptake at 4°C and at 37°C suggested that internalization of MP-oligonucleotides occured by an active mechanism. To make sure that the uptake process was not governed by the presence of the additional charged 5' phosphate moiety in the [^{32}P] labeled MP-oligonucleotides, we also examined uptake of TRITC labeled MP-oligonucleotides, as well as uptake of free (unconjugated) TRITC as a control. Uptake of TRITC labelled MP-oligonucleotides and of free TRITC were quantified by digitized video imaging as described in the Methods section. Fig 1B shows the temperature dependence of uptake of TRITC labelled MP-oligonucleotides. As with the uptake of [^{32}P] labeled MP-oligonucleotides, uptake of TRITC labeled MP-oligonucleotides was significantly lower at 4°C than at 37°C. By contrast, uptake of free TRITC was not temperature dependent. Since two different labelling methods showed temperature dependent cellular accumulation, it seems likely that MP-oligonucleotides are taken up by an active cellular process rather than by passive diffusion across the cell membrane. It is not clear why the uptake plateaus at 10−12 hrs.; this may represent exocytosis of the oligonucleotide, or other loss from the cell, but for MP-oligonucleotides it probably does not represent degradation (see below).

In Fig 2 the temperature dependence of cellular uptake of [^{32}P] labelled MP-oligonucleotides is analyzed in more detail, with measurements taken at intervals of a few degrees. The uptake profile shows a sharp discontinuity between 15 and 20°C. This is very characteristic of active endocytosis, but is distinct from the expected temperature profile for passive diffusion which would be likely to exhibit a gradual increase [7, 26].

Cell surface binding of MP-oligonucleotides

The cell associated [^{32}P] MP-oligonucleotides in Fig.1 could be due to surface binding as well as to uptake into intracellular compartments. In order to asses the fraction of cell associated MP-oligonucleotides actually within the cell, low pH buffer or trypsinization were used to strip off cell surface bound material [19]. Fig. 3 shows the effect of these treatments on residual levels of cell associated [^{32}P] MP-oligonucleotide. After incubation at 37°C, the bulk of the cell associated [^{32}P] MP-oligonucleotide is refractory to removal by low pH or trypsin, indicating that it is within the cell; about 20−30% of the label was removed by these treatments and thus seems to be on the cell surface. By contrast, when cells were incubated at 4°C, and are thus unable

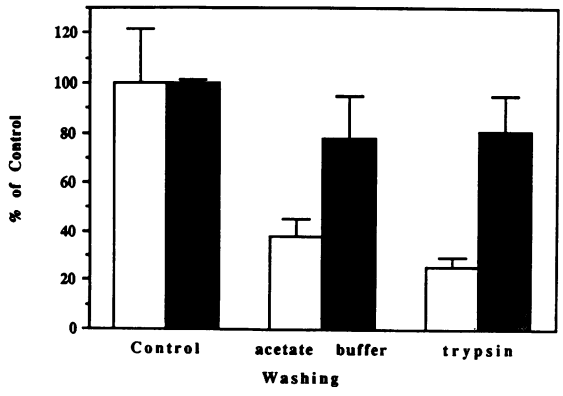

Fig. 3. Intracellular uptake of methylphosphonate oligodeoxynucleotides. Cells were incubated with 0.01μM MP-oligonucleotides at 4°C (clear) or 37°C (solid) for 6 hours in HEPES buffer. After the incubation cells were washed with acetate buffer (pH 2.3) as described in Methods. Trypsin was added to cells as an alternative method to strip off surface bound MP-oligonucleotides. Cell-associated radioactivites were determined as described in Methods. Amounts of uptake were expressed as percentage of that of control at each temperature. Bars are standard deviations. Experiments were carried out in triplicate.

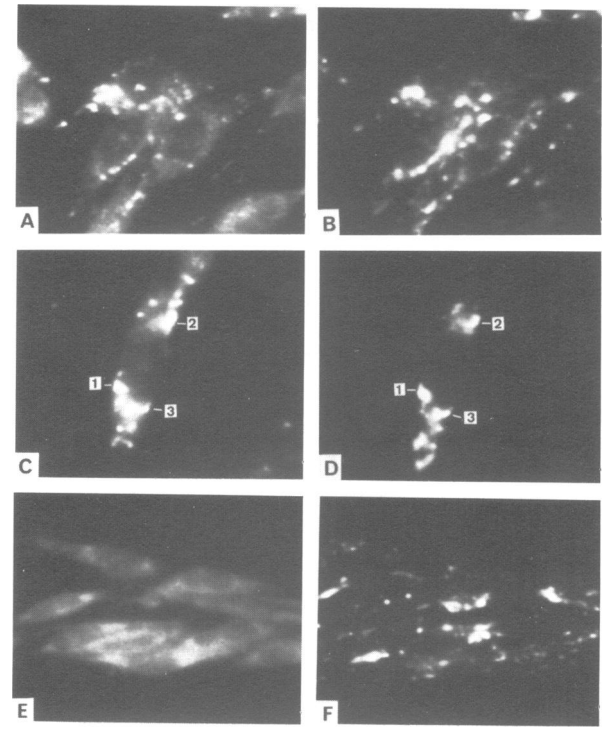

Fig. 4. Subcellular distribution of TRITC labeled methylphosphonate oligodeoxynucleotides. CH^{R}C5 cells were pre-incubated with 2mg/ml FITC labelled dextran at 37°C for 1 hour in complete alpha-MEM medium, washed and then incubated overnight. Cells pretreated with dextran were incubated with 4μM TRITC labeled MP-oligonucleotides in HEPES buffer (pH 7.4) at 37°C for 1hour. Rhodamine and FITC images were monitored using the digitized imaging system. (A) TRITC labeled MP-oligonucleotides; (B) corresponding image with FITC dextran; (C) TRITC labeled MP-oligonucleotides; (D) same cell with FITC dextran; (E) cells with free TRITC; (F) same cells with FITC dextran. Numbers in C, D show points of coincidence of the TRITC and FITC images.

to take up material by endocytosis, almost all of the cell associated material could be removed by low pH or trypsin treatments. A companion study [28] shows that MP-oligonucleotides are strongly bound to lipid membranes; thus the presence of adsorbed MP-oligonucleotide on the cell surface is not surprising.

Subcellular distribution of MP-oligonucleotides

Subcellular distribution patterns of TRITC labeled MP-oligonucleotides were visualized by digitized video imaging. As seen in Fig 4A, TRITC labelled MP-oligonucleotides showed a punctate distribution with a tendency to accumulate in the perinuclear region. There was also some diffuse fluorescence apparently in the cytoplasm. Only a small amount of fluorescence was observed in the nuclear region. The distribution of TRITC labeled MP-oligonucleotides was compared, in the same cells, with that of FITC labelled dextran, a marker of endocytic vesicles and lysosomes[22,29]. Fig. 4B shows a partial co-distribution of TRITC labeled MP-oligonucleotides with FITC-dextran, with numerous vesicles found to contain both markers. This indicates that some MP-oligonucleotides were internalized by the same endocytotic pathway as dextran. This co-distribution was observed up to 24 hours (data not shown), suggesting that intact MP-oligonucleotides stay in lysosomal vesicles. This is consistent with previous observations on the permeability characteristics of endosomes/lysosomes, which suggest that only rather small molecules can diffuse across the organelle membrane and enter the cytoplasm [30]. The numbered arrows in Fig 4 C,D show vesicles containing both TRITC labeled MP-oligonucleotides and FITC-dextran; numbers are matched in both images. The intracellular distribution of MP-oligonucleotides and dextran were not entirely coincident, however, since the diffuse fluorescence observed with the TRITC labeled MP-oligonucleotides was not observed with the FITC-dextran. In addition, some vesicular structures seemed to contain primarily one label or the other. At this point it is not clear if the diffuse fluorescence obtained with the TRITC labeled MP-oligonucleotides is due to oligonucleotide in the cytoplasm, or oligonucleotide adsorbed to the cell surface; as reported elsewhere [28], MP-oligonucleotides can bind strongly to membranes. As shown in Fig.4D, free TRITC was diffusely distributed into cytoplasmic regions with a distinctively different distribution pattern than TRITC labeled MP-oligonucleotides. Since FITC labeled dextran did not co-localize with free TRITC, it seems clear that free TRITC was not accumulated into endocytotic vesicles (Fig.4E). Control experiments showed that no fluorescence was detected in FITC loaded cells when the rhodamine filters were used, and no fluorescence was detected in TRITC loaded cells when the fluorescein filters were used.

Inhibition of uptake of MP-oligonucleotides

These experiments were carried out to determine whether specific binding sites are involved in the uptake of MP-oligonucleotides, as has been sugested for the uptake of phosphodiester oligodeoxynucleotides [8,9]. Different concentrations of potential competitors including unlabled D-oligonucleotides, MP-oligonucleotides, or ATP were pre-incubated with cells for 15 min at 37°C. After pretreatment, [^{32}P] labelled MP-oligonucleotides were incubated for 3 hours at 37°C in the continued presence of competitors. Cell viability was monitored by trypan blue staining and no toxicity was observed. Fig. 5A shows the effects of several potential competitors on cellular uptake of [^{32}P] MP-oligonucleotides. ATP did not significantly inhibit uptake of MP-oligonucleotides even at very high concentration (10mM). Inhibition of uptake of [^{32}P] MP-oligonucleotides by unlabeled D-oligonucleotide or MP-oligonucleotide was also modest, with significant effects observed only at a 1000-fold excess. We also tested the ability of various potential competitors to inhibit the uptake of TRITC labeled MP-oligonucleotides. As seen in Fig 5B neither MP-oligonucleotides nor D-oligonucleotides effectively inhibited uptake of TRITC labled MP-oligonucleotides. By contrast, uptake of [^{32}P] labeled D-oligonucleotide was completely inhibited by unlabeled D-oligonucleotides in a dose dependent manner (Fig.5C), confirming observations of other investigators [5]. These results confirm previous observations suggesting that the uptake of D-oligonucleotides involves specific cell surface binding sites which are subject to competition. By contrast, the uptake of MP-oligonucleotides does not seem to involve saturable cell surface binding sites, and may thus be due

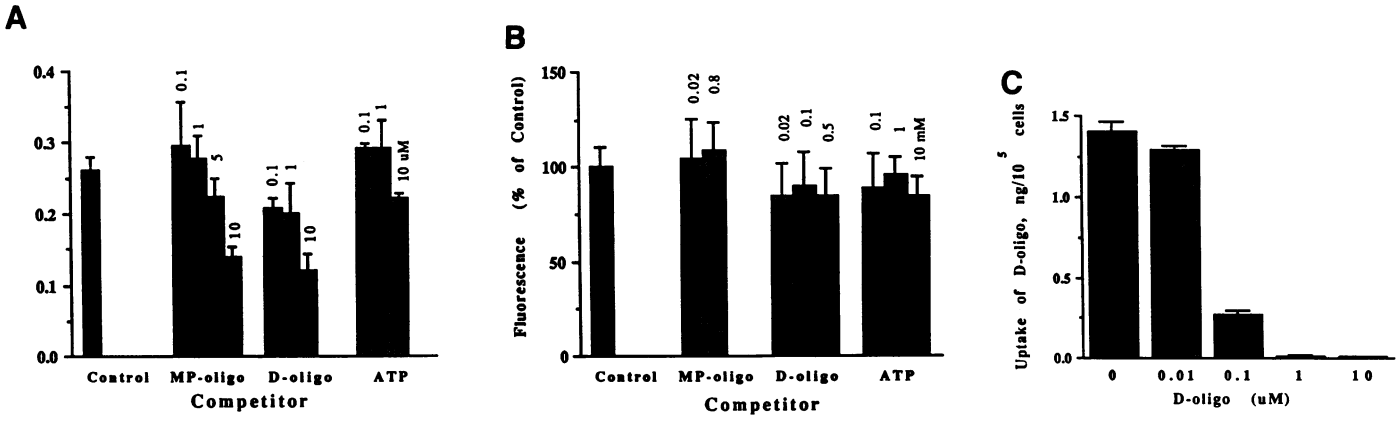

Fig. 5. Lack of inhibition of uptake of MP-oligodeoxynucleotides by competitors. Cells were incubated with competitors and radiolabeled D-, or MP-oligonucleotides as described in Methods. (A) Inhibitory effects on uptake of [^{32}P] labelled MP-oligonucleotides. Cells were incubated with 0.01μM [^{32}P] labeled MP-oligodeoxynucleotide in alpha-MEM containing 10% heat inactivated FCS for 3 hour along with inhibitors as indicated. (B) Inhibitory effects on uptake of TRITC labelled MP-oligonucleotides. TRITC labeled MP-oligodeoxynucleotides (4μM) were added to cells and incubated in HEPES buffer for 1 hour along with inhibitors as indicated. (C) Dose dependent inhibition on uptake of D-oligonucleotides. Cells were incubated with 0.01μM [^{32}P] labeled D-oligodeoxynucleotide in HEPES buffer for 3 hours along with excess unlabeled D-oligonucleotides. The numbers in A represent the concentration of inhibitor in μM; in B the concentation of inhibitor is in mM. Bars are standard deviations.

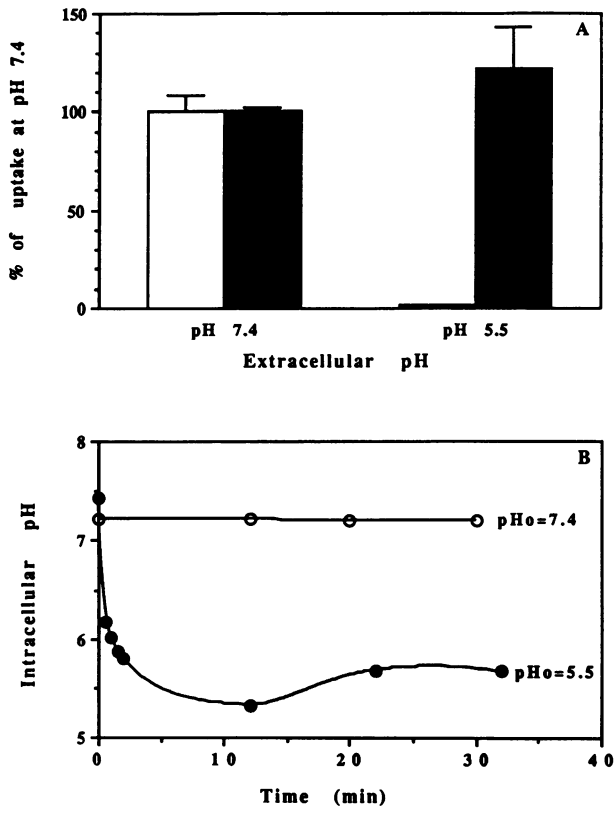

Fig. 6. Effect of acidification of cytosol on uptake of methylphosphonate oligodeoxynucleotides. Cells were incubated in alpha-MEM containing 0.1% BSA and buffered with HEPES (pHo=7.4) or MES (pHo=5.5) at 37°C for 30 min. (A) shows the effect of acidification of cytosol on uptake of D, or MP-oligonucleotides. Each experiment is in triplicate. Bars are standard deviations. D-oligonucleotides (open); MP-oligonucleotides (solid). (B) The intracellular pH was measured using BCECF-AM loaded cells and the digitized imaging system under both neutral pHo and acidic pHo conditions.

to fluid phase endocytosis or to non-specific adsorbtive endocytosis [26,27,29].

Inhibition of receptor-mediated endocytosis

Since acidification of cytoplasmic pH inhibits receptor-mediated endocytosis but not fluid phase endocytosis [24,27], the pathway of internalization of MP-oligonucleotides was evaluated by incubating the cells at normal or acidic cytoplasmic pH and comparing the uptake of [^{32}P] labeled MP oligonucleotides. As shown in Fig.6B, cytosolic pH rapidly dropped (within 2min) to around pH 5.7 when the external pH was changed to pH 5.5. Cells were pre-incubated in HEPES-MEM (pH 7.4) or MES-MEM(pH 5.5) buffer for 5 min prior to addition of [^{32}P] labelled MP-oligonucleotides; cells were then incubated at 37°C for 30 min. As shown in Fig. 6A, uptake of D-oligonucleotides in cells was completely inhibited when the cytosol was made acidic. By contrast, uptake of MP-oligonucleotides was not inhibited by acidification of the cytosol.

Lack of degradation of MP-oligonucleotides in cells

Our laboratory previously demonstrated that MP-oligonucleotides were quite stable under tissue culture conditions while D-oligonucleotides and S-oligonucleotides were degraded in 3 hours [31]. The stability of intracellular MP-oligonucleotides is still

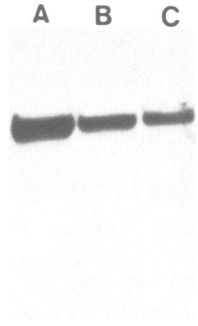

Fig. 7. Recovery of MP-oligonucleotides from cells and medium. [^{32}P] labelled MP-oligonucleotides were incubated with cells for 3 hrs at 37°C. Radiolabeled material was recovered from cells and from culture medium as described in Methods. The material was analyzed by denaturing PAGE and autoradiography. A. Original oligonucleotide. B. Oligonucleotide recovered from medium. C. Oligonucleotide recovered from cells.

unknown. To elucidate whether intracellular MP-oligonucleotides are degraded or not, 5′-[^{32}P] labelled MP-oligonucleotides were recovered from cells and were analyzed by denaturing gel electrophoresis (Fig.7). After 3 hours incubation at 37°C, intact 15 mer MP-oligonucleotides were recovered from the medium (lane B) and from cells (lane C) with no degradation. By contrast, the majority of D-oligonucleotide was rapidly degraded within 3 hours in medium or cells (data not shown).

DISCUSSION

In this report we used two different labels to investigate the mechanism of cellular transport of oligodeoxynucleotides containing methylphosphonate linkages. In contrast to early reports which suggested that MP-oligonucleotides enter cells by passive diffusion [13], we found that the dominant mechanism of cellular uptake of MP-oligonucleotides seems to be via a fluid phase/adsorbtive endocytotic route. This is also supported by work in a companion report [28] showing that MP-oligonucleotides cannot passively diffuse across phospholipid membranes at significant rates. The uptake pathway for MP-oligonucleotides appears to be distinct, however, from that for phosphodiester or phosphorothioate oligodeoxynucleotides which seem to enter cells by endocytosis involving a saturable binding site.

The MP-oligonucleotides used this study proved to be quite stable both in culture medium and within cells. Thus the uptake measurements reflect the behavior of the intact molecule and not breakdown products. Since the nature of the 'tag' used to follow uptake of an oligodeoxynucleotide might affect its behavior, we used both [^{32}P] and TRITC to label the MP-oligonucleotides. Both labels provided similar results, indicating that the uptake process was determined by the chemical characteristics of the oligodeoxynucleotides and not that of the label. The use of fluorescent TRITC labeled oligodeoxynucleotides also permitted visualization of their intracellular distribution.

MP-oligonucleotides labeled with either ^{32}P or TRITC showed a discontinuous temperature dependence of cellular

uptake (Fig.1, 2), which is characteristic of endocytosis but not of passive diffusion [6,7,26]. However, uptake of labeled MP-oligonucleotides was not readily competed by excess unlabeled material, indicating that it is unlikely that specific cell surface binding sites are involved in the endocytotic process (Fig.5). This contrasts with the case of phosphodiester oligodeoxynucleotides where excess unlabeled material blocks uptake of radiolabeled oligodeoxynucleotides. Thus the uptake process for MP-oligonucleotides may be fluid phase or adsorbtive endocytosis, but is unlikely to be receptor mediated endocytosis. This interpretation is also supported by experiments using acidification of cytoplasmic pH to block receptor mediated endocytosis [27]. Sandvig et al [24] reported that cytoplasmic acidification inhibited uptake of epidermal growth factor and transferrin, but did not strongly affect uptake of ricin or lucifer yellow, markers of fluid-phase endocytosis, indicating that a pH insensitive pathway is responsible for fluid phase/adsorbtive endocytosis. Uptake of D-oligonucleotide but not of MP-oligonucleotide was strongly blocked by acidification of the cytoplasm (Fig 6). This result clearly shows that the pathway of cellular uptake of MP-oligonucleotides is distinct from that of D-oligonucleotides. Our data also suggest that uptake of MP-oligonucleotides may involve the fluid/adsorbtive endocytotic pathway defined by markers such as lucifer yellow. Based on the fact that MP-oligonucleotides bind strongly to membranes [28], adsorbtive endocytosis is likely to be important. Thus, based on our work in CHO cells, there seems to be more than one pathway for active endocytotic uptake of oligonucleotides; however, the generality of these effects in different cell types remains to be determined.

It should be noted that the oligonucleotides used in this study contain 14 methylphosphonate linkages but retain a single phosphodiester linkage and thus have a charge. It is conceivable that oligonucleotides with all methylphosphonate linkages might enter cells by a completely different mechanism such as passive diffusion. However, we feel that this is unlikely. Although an uncharged, fully methylphosphonate oligonucleotide may have a greater oil-water partition coefficient than an oligonucleotide with a single charge, the diffusion rate of such a large molecule across the membrane bilayer is still likely to be very low [7,32,33].

Use of a digitized video imaging system allowed visualization of the intracellular distribution of TRITC labeled MP-oligonucleotides. The distribution pattern suggested that the MP-oligonucleotides were primarily confined to vesicular structures, although some diffuse cytoplasmic fluorescence was also observed; for the most part the nucleus remained dark indicating little redistribution to that site during the course of the experiment. The distribution pattern of TRITC labeled MP-oligonucleotides overlapped substantially with that of fluoresceinated dextran which is considered to be a good marker for endosomes and lysosomes [22]. The fluorescence pattern observed for TRITC labeled MP-oligonucleotide is unlikely to result from the redistribution of free TRITC cleaved from the oligodeoxynucleotides, since quite a different pattern was observed when the cells were incubated with free TRITC, one which did not show co-localization with dextran. Thus the morphological evidence also supports the concept that MP-oligonucleotides are primarily taken up by endocytosis and accumulate in an endosomal/lysosomal compartment.

Recently two groups have independently reported that oligonucleotides microinjected into the cytoplasm, including MP-oligonucleotides, were rapidly accumulated into the nucleus [34,35]. In our studies, if MP-oligonucleotides escaped from the lysosomal-endosomal compartment into the cytoplasm to any great extent, then we should have observed redistribution to the nucleus and strong nuclear fluorescence. Since this did not occur, it seems likely that only a small portion of the MP-oligonucleotide which accumulates in the cell gains entry into the cytoplasm. This has interesting implications for the pharmacological actions of oligonucleotides, suggesting that only a tiny fraction of the cell associated material is responsible for the full pharmacological effects. It also suggests that endosome to cytoplasm transfer may be a critical limiting step for the pharmacological actions of highly charged oligonucleotides such as D-oligonucleotides and S-oligonucleotides, as well as for chemically modified oligonucleotides containing nonpolar substituents, such as MP-oligonucleotides, since both types accumulate in the endosomal compartment. Thus delivery strategies which facilitate endosome to cytosol transfer may be of value in the successful therapeutic application of oligonucleotides.

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Note added

Following submission of this work we became aware of a recent publication indicating heterogeneous uptake of oligonucleotides in lymphoid cells; only some of the uptake was completed by DNA, thus suggesting fluid phase endocytosis.

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