Cellular Delivery of Oligonucleotides by Synthetic Import Peptide Carrier

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Purpose. Inefficient cellular uptake and endosomal entrapment are among the obstacles impeding the therapeutic use of oligonucleotides (ONs). The objectives of this study are to investigate the feasibility of utilizing a synthetic import peptide as a drug carrier for cytoplasmic delivery of ONs and to study its transport mechanisms.

Methods. A molecular conjugate consisting of a signal import peptide (IP) derived from Kaposi fibroblast growth factor (K-FGF) and a polycationic ON linker, polylysine (PL), was synthesized and complexed with 5′ fluorescently-labeled ON. Complex formation was verified by spectral shift assay and cellular uptake of the ON complex was studied fluorometrically. Microscopic studies were performed to visualize the intracellular distribution of the ON.

Results. Cells treated with the ON:IP-PL complex exhibited a dose-dependent increase in ON uptake over free ON-treated controls. The uptake of the complex was shown to occur via an energy-independent, non-endocytic, process since metabolic and endocytic inhibitors and low temperature did not prevent the uptake. Microscopic studies revealed a non-punctate fluorescence pattern, consistent with the non-endocytic transport process. Intense nuclear fluorescence was observed in cells treated with the complex but not with free ON, suggesting enhanced cytoplasmic delivery and nuclear accumulation of the ON by the conjugate. Efficient complex uptake was shown to require both the ON-binding moiety PL and the IP moiety. The delivery system was found to be non-toxic at the concentrations used.

Conclusions. The peptide carrier was effective in promoting the cellular uptake of ON. The mechanism by which the peptide facilitates ON uptake appears to involve a direct translocation of ON via a non-endocytic process. The peptide carrier has the potential to overcome the problem of ON endosomal entrapment and degradation.

KEY WORDS: oligonucleotide; uptake; delivery; endocytosis; signal peptide.

INTRODUCTION

A major problem encountered with the therapeutic use of oligonucleotides (ONs) is low cellular permeability, a characteristic that is due to the physicochemical properties of the compounds, i.e., large molecular size and high charge density. Nonetheless, ONs have been shown to be taken up by cells to some extent via an energy-dependent endocytic process (1–3). This mode of uptake, however, results in the ONs being trapped in endocytic vesicles and eventally degraded in lysosomes. In order to improve the cellular delivery of ONs, several methods have been developed which include the use of liposomes (2), viral vectors (3), and receptor-mediated endocytosis (4). Although these methods have been shown to be beneficial, they facilitate ON uptake via an endocytic process, thus the problem of endosomal entrapment remains. To overcome this problem, several strategies including the use of pH-sensitive lipids (5,6) and fusogenic peptides (7,8) have been investigated.

In this study, we propose an alternative approach for direct cytoplasmic delivery of ONs using a synthetic import peptide (IP). The IP is derived from naturally occurring signal peptide (SP). This method utilizes the hydrophobic region of the signal peptide K-FGF as the membrane-translocating carrier (9). This segment of the SP is known to interact with lipid bilayers (10). We rationalize that this region, known to facilitate secretion of proteins, can be applied to import macromolecules to the inside of a cell. To serve as an ON carrier, the peptide is covalently conjugated to a polycationic linker, poly-L-lysine (PL), which can then complex electrostatically with the polyanionic backbone of the ON. We tested the potential enhancing effect of the IP-PL conjugate on ON uptake in various cell lines. The mechanism of IP-PL-mediated ON uptake was further investigated in human adenocarcinoma A549 cells.

METHODS

Oligonucleotide and Peptide Synthesis

Nuclease-resistant phosphorothioate ON with a sequence complementary to the mutated codon 12 of K-*ras* oncogene (G->T) (5′-GCCTCTAGCT-3′) was synthesized on an automated solid-phase synthesizer using standard phosphoramidate chemistry (Oligo Therapeutics Inc., Wilsonville, OR). A fluorescent label was attached to the terminal 5′-linkage group by the use of 5′-carboxyfluorescein phosphoramidate. The ON was purified by high performance liquid chromatography (HPLC) and was >98% pure. The peptide import carrier consisting of the hydrophobic sequence of K-FGF signal peptide (AAVALLPAVLLALLAP) and a 10-mer poly-L-lysine was synthesized using the Fmoc procedure (Quality Controlled Biochemicals, Hopkinton, MA). The peptide was purified by HPLC and verified by mass spectrometry.

Preparation of IP-PL:ON Complex

The complex was formed by mixing a 1:1 volume ratio of the labeled ON (10 μM) with IP-PL (10–500 μM) in Dulbecco's Modified Eagle Medium (DMEM). The solution was incubated at 37°C for 30 min before use. Complex formation was verified by spectral shift assay using scanning fluorescence spectroscopy. The addition of IP-PL to the ON-containing medium resulted in a shift of the ON emission peak from 519 ± 1 nm to 526 ± 1 nm at the maximum excitation wavelength of 490 nm. No significant change in the fluorescence intensity of the ON was observed upon addition of the IP-PL. The IP-PL or the medium alone showed no fluorescence emission peak between 350–1000 nm at the maximum excitation wavelength of 490 nm. Thus, these results indicated that the complex was indeed formed under the experimental conditions. In a separate study,

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the stability of the ON complex or ON alone in DMEM medium containing 10^6/ml cells was also evaluated using gel electrophoresis. After a 3 h incubation period at 37°C the samples were centrifuged and the supernatants were electrophoresed in 20% w/v polyacrylamide-7M urea gels and analyzed by fluorography. No detectable degradative products of ON were observed in this study.

Cell Culture

The adenocarcinoma A549 cells (ATCC# 185-CCL) were obtained from the American Type Cell Culture Collection (Rockville, MD). The cells were grown in F-12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 μg/ml streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Prior to use, the cells were briefly trypsinized or mechanically scraped and centrifuged. The cell pellet was rinsed three times and resuspended with serum-free DMEM medium. The other cell lines, SV40, P39, and RAW264.7, were obtained from the National Cancer Institute (Bethesda, MD) and were cultured under similar conditions using the same medium.

Cellular Uptake Studies

Cells (1×10^6/ml) were incubated with fluorescein-labeled ON (10 μM) at 37°C (or 4°C where indicated) in the presence or absence of IP-PL conjugate (100 μM). In studies designed to evaluate the effect of concentration of the peptide conjugate on ON uptake, various concentrations of the peptide (10–500 μM) were used. After a specified period of incubation, the cells were centrifuged at 1,500g for 10 min and the supernatants were collected and analyzed fluorometrically at the excitation/emission wavelengths of 490/520 nm. The cell pellets were resuspended and treated with trypsin or pronase (1 mg/ml, 5 min) to remove surface-bound ON. They were then washed with fresh medium, lysed with 0.1% Triton-X, and measured for their fluorescence intensities. In studies designed to assess the transport mechanisms of the ON complex, sodium azide (1 mM), cytochalasin B (0.1 mM), or unconjugated peptide (IP) (1 mM) were also added to the incubating medium 1 h prior to the experiments. To assess the potential membrane channel formation induced by the peptide, two hydrophilic probes, carboxyfluorescein and ^{3}H mannitol, were used. These compounds, at 1 μg/ml and 0.1 μCi/ml respectively, were added to cells in the presence of IP-PL conjugate, and their cellular uptake was determined fluorometrically and radioactively.

Fluorescence Microscopy

Cells were incubated with ON or ON:IP-PL complex according to the procedure described above. After appropriate time intervals, e.g., 5, 10, 30, 60, 120, and 180 min, the cell samples were washed in trypsin containing medium and placed on glass cover slips. They were immediately observed under a fluorescence microscope (IM-35, Carl Zeiss Inc.) at the excitation/emission wavelengths of 490/520 nm. The images were captured through a CCD-72 camera (Gen11sys, Dage-MIT Inc.) and digitized in an imaging system (IP512, Imaging Technology, MA).

MTT Assay

MTT assay was used to indicate the viability and proliferation of cells in our experiments. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) was dissolved in PBS at 5 mg/ml. At the times indicated, stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, the plates were read on a Bio-Rad Microplate reader (Model 550), using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Reversibility Studies

Cell suspension was pretreated with IP-PL (100 μM) for 3 h at 37°C. The cells were then washed three times with DMEM and incubated in the same medium for an additional 1, 6, 12 h at 37°C to allow for cell recovery. After the recovery period, the cells were incubated with ON (10 μM) for 1 h. Subsequently, the cells were washed and centrifuged at 1,500g for 10 min. The cell pellets were washed, lyzed with 0.1% Triton-X, and measured for fluorescence intensities. As a control, cells were incubated with ON for 1 h without any IP-PL treatment and the fluorescence intensity was similarly determined.

RESULTS

Cellular Uptake of Free and Complexed Oligonucleotides

Cellular uptake of free ON and complexed ON with varying amounts of IP-PL is shown in Fig. 1. The results indicate

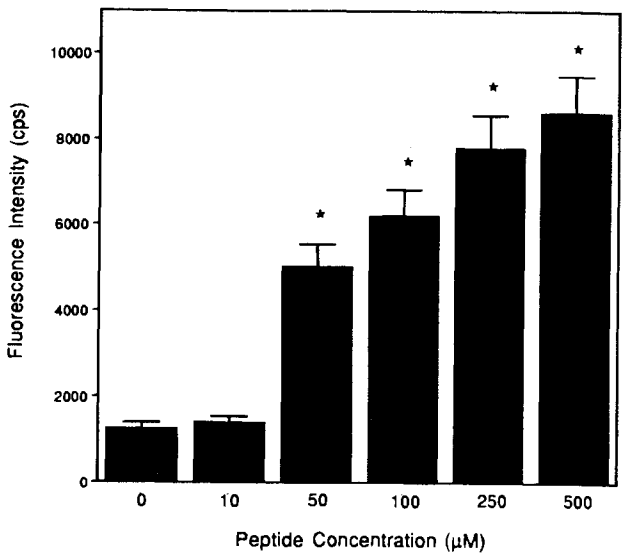

Fig. 1. Dose-Dependent Uptake of ON Mediated by IP-PL Conjugate. A549 cells (1×10^6/ml) were incubated with ON (10 μM) in the presence of increasing concentrations of IP-PL (10–500 μM) in DMEM for 3 h at 37°C. After incubation, the cells were washed and analyzed for cellular fluorescence at the excitation and emission wavelengths of 490 nm and 520 nm. The data represent mean ± SE of four measurements after corrected for background autofluorescence. * indicates significant difference over free ON-treated control ($p < 0.05$).

that the peptide import carrier can significantly enhance cellular uptake of ON in A549 cells. Complexation with the IP-PL conjugate resulted in a dose-dependent increase in cellular ON fluorescence as compared to the free ON-treated control. To test whether the IP-PL can promote the cellular uptake of ON in other cell lines, uptake studies were repeated using the macrophage RAW264.7, epithelial P39 and SV40 cell lines. In all cell lines tested the uptake of the ON in the presence of IP-PL was significantly higher than that of the free ON (Fig. 2). The magnitude of enhancement varied with the cell type with the rank order of uptake being P39 > A549 > SV40 > RAW264.7. In the absence of IP-PL, the uptake of ON in all cell types was low and comparable. These results indicate that the IP-PI conjugate system is not only effective in the A549 cell line but is also useful in other cell lines. The basis for the observed difference in cellular uptake is not yet clear but is probably due to the difference in cellular interactions with the IP-PL. The observed low uptake of the ON:IP-PL complex in the macrophage cell line, which can be assumed to possess a high endocytic activity due to its phagocytic origin, suggests that the uptake of the complex may not be mediated by an endocytic process. Subsequent uptake studies further confirm this point.

Structural Requirement of IP-PL-Mediated ON Uptake

To investigate the structural requirement of the IP-PL conjugate in promoting ON cellular uptake, the contribution of each domain of the conjugate was evaluated. The ON in the presence of unconjugate IP, PL, or the combination was evaluated for their ability to promote cellular uptake of ON in comparison to that of the IP-PL conjugate (Fig. 3). The complex consisting of the ON and IP-PL conjugate was taken up more efficiently by cells than the complexes consisting of ON in combination with IP, PL, or IP and PL. These results demonstrated that effective ON uptake mediated by the conjugate required functional domains capable of both ON binding and cell surface association. Polylysine by itself is also known to increase the cellular uptake of ON (11). However, this effect is cell type dependent. Our results showed that polylysine did not have an enhancing effect on ON uptake in the A-549 cell line. A possible explanation for this observation is that in our study a short polylysine (10-mer) was used, whereas in most other studies which demonstrated ON uptake enhancement, high molecular weight polylysine (MW >10,000) was used. Thus, the above results confirm that the peptide and not the polylysine is responsible for the enhancing effect and that polylysine purely acts as an ON linker. The partial enhancing effect of IP on ON uptake may be attributed to the non-specific interaction between IP and ON, which could result in an increased cellular association of the ON.

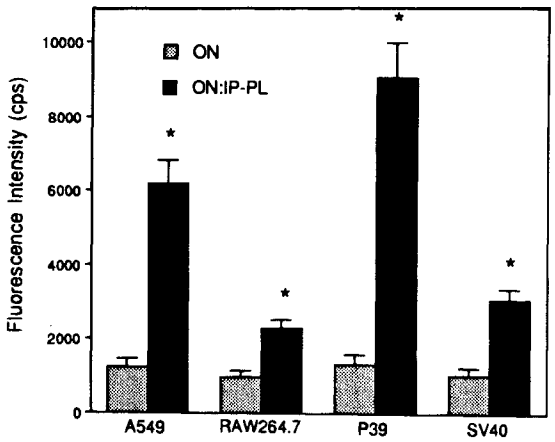

Fig. 2. Cell Type-Dependent Uptake of ON Mediated by IP-PL. Cells (1×10^6/ml) were incubated with ON (10 μM) in the presence or absence of IP-PL (100 μM) in DMEM medium at 37°C for 3 h. The cell lines used were A549, P39, SV40 and RAW264.7. * indicates significant difference over free ON-treated controls ($p < 0.05$, n = 4).

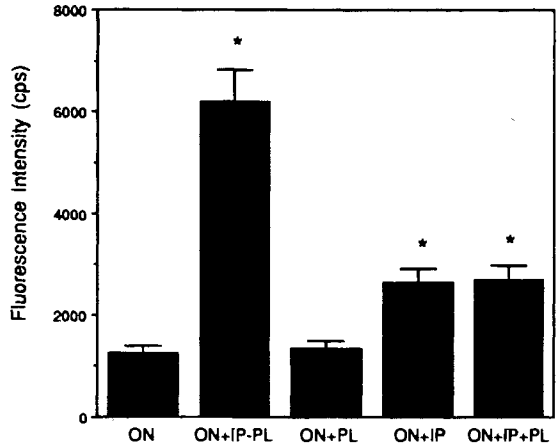

Fig. 3. Structural Requirement of IP-PL-Mediated ON Uptake. Cells were incubated with ON (10 μM) in the presence of 100 μM of IP-PL, IP, PL, or the combination of IP and PL in DMEM medium at 37°C for 3 h. * indicates significant difference over free ON-treated control ($p < 0.05$, n = 4).

Microscopic Studies of Oligonucleotide Uptake

To provide morphologic evidence of ON uptake, cells were incubated with free ON or ON:IP-PL complex at 37°C for different time periods and examined for their intracellular ON distribution using fluorescence microscopy. As seen in Fig. 4A-D, cells treated with ON:IP-PL complex exhibited a strong fluorescence intensity, whereas those treated with free ON showed a very weak fluorescence signal. At 10 min after treatment (Fig. 4B), the ON:IP-PL-treated cells showed appreciable nuclear fluorescence distribution. Some diffusely distributed fluorescence was also apparent in the cytoplasm. At latter time points, the nuclear fluorescence intensity was further enhanced (Fig. 4C), suggesting increased cytoplasmic entry and nuclear localization of the ON. These results indicated that the IP-PL conjugate was able to facilitate the cellular entry of ON and that the ON by itself was poorly taken up by cells. The lack of punctate fluorescence pattern in the cytoplasm, characteristic of endocytic uptake, suggested a non-endocytic transport of the IP-PL-mediated ON uptake.

Mechanism of Oligonucleotide Uptake Mediated by IP-PL

To further confirm the non-endocytic uptake mechanism, experiments were carried out at 4°C, a condition known to inhibit endocytosis. Under this condition, uptake was not inhibited (Fig. 5). Treatment of the cells with an endocytic inhibitor, cytochalasin B, or a metabolic inhibitor, sodium azide, did not

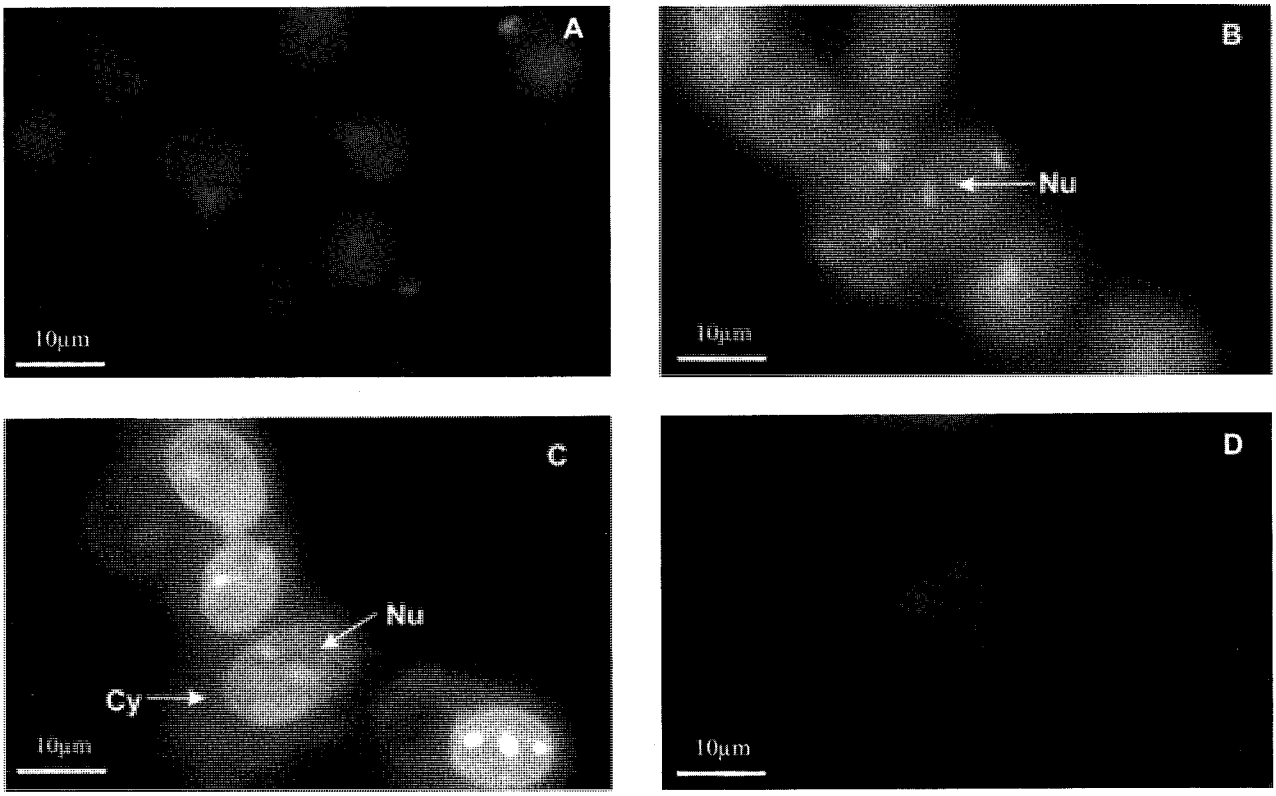

Fig. 4. Fluorescence Micrographs of A549 Cells Showing Cellular Distribution of Oligonucleotide. Cells were incubated with ON (10 μM) in the presence of IP-PL (100 μM) for 5 min (A), 10 min (B), or 1 h (C) at 37°C in DMEM medium. (D) indicates ON-treated cells (10 μM) for 1 h at 37°C in the absence of IP-PL. Nu = nucleus and Cy = cytoplasm.

significantly affect the uptake of the complex (Fig. 5). These studies confirmed the non-endocytic nature of the process. Furthermore, in the presence of an excess amount of IP, the uptake of the complex was again unaffected, indicating that the IP could not compete for the complex uptake and that this uptake was not receptor mediated.

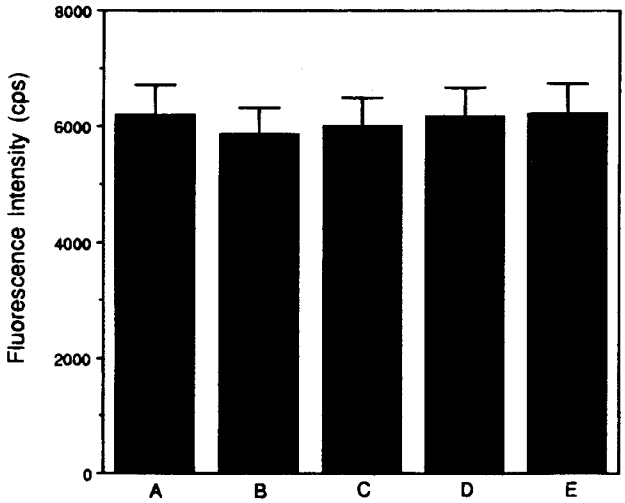

Fig. 5. Mechanism of ON Uptake Mediated by IP-PL conjugate. Cells were incubated with ON (10 μM) in the presence of IP-PL (100 μM) for 3 h at 37°C (A) or 4°C (B). Experiment at 37°C was repeated but in the presence of 1 mM sodium azide (C), 0.1 mM cytochalasin B (D), or 1 mM unconjugate IP (E). No significant difference was seen when the different groups were compared ($p < 0.05$, n = 4).

The observation of non-endocytic uptake of the complex leads to an intriguing question as to the precise mechanism of ON uptake mediated by the conjugate. To examine this, we first investigated the possible cytotoxic or damaging effect of the IP-PL which may lead to enhanced ON uptake. In this study, the cells were treated with varying concentrations of IP-PL (25–100 μM) for 3 h at 37°C, after which they were analyzed for potential cytotoxicity using the MTT assay. Our results indicated that, at all concentrations used, the IP-PL did not cause any detectable toxic effects to the cells (Fig. 6).

Because the IP-PL peptide is derived from SP and because SPs are known to facilitate the transport of many secretory proteins across membranes through hydrophilic protein-conducting channels (9,10), we therefore investigated the possibility of channel formation induced by our peptide conjugate. We used two small hydrophilic probes, ^{3}H mannitol (M) and carboxyfluorescein (CF) as indicators for channel formation. Due to their hydrophilic nature, these compounds are normally excluded from cells. However, if the channel or pore formation occurs, they would be able to enter the cells. Thus, increased intracellular M radioactivity or CF fluorescence indicates channel/pore formation. An addition of M or CF to the cells in the presence of the IP-PL conjugate resulted in a modest but significant increase in cellular M and CF activity (Fig. 7). The magnitude of uptake enhancement was much lower than that observed with the larger ON under identical conditions (MWs

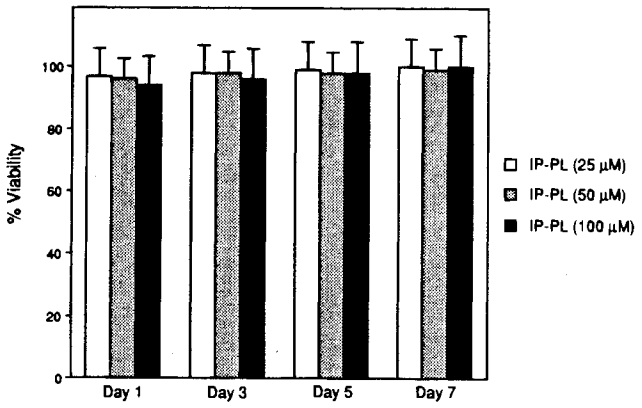

Fig. 6. Toxicity of ON:IP-PL on A549 cells. Cells were plated in 96-well plates. They were treated with different concentrations of IP-PL (25–100 μM) for 3 h at 37°C. After incubation, the cells were washed free of peptide and allowed to grow for 1, 3, 5, and 7 d. MTT assay was then performed on the cultured cells. No significant difference was seen among groups ($p < 0.05$, n = 3).

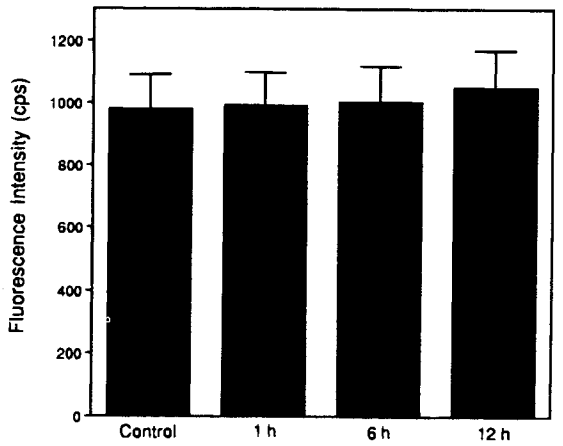

Fig. 8. Reversibility of the IP-PL Effect. A549 cells were pretreated with IP-PL (100 μM) for 3 h at 37°C. After treatment, the cells were washed and incubated in DMEM for an additional 1, 6, 12 h at 37°C to allow for cell recovery. After the recovery period, the cells were incubated with ON (10 μM) for 1 h, followed by fluorescence measurements. As a control, cells were incubated with ON for 1 h without any IP-PL treatment. No significant difference was seen among groups ($p < 0.05$, n = 3).

of M, CF, and ON are 180, 375, and 3,100 respectively). Thus, it is possible that a different transport mechanism is responsible for the IP-PL-mediated ON uptake, or alternatively the channels formed by the peptide may be selective to the transport of the ON compared to M or CF, i. e., due to the close proximity of the ON to the peptide.

Reversibility Studies

The potential for channel formation induced by the peptide raises a concern regarding its long-term effect on cell permeability and functions. We carried out an experiment to test whether this pore/channel formation is temporary or permanent. We first treated the cells with IP-PL for 3 h, after which they were thoroughly washed and incubated in fresh medium for various periods to allow for cell recovery. At the end of each incubation period, the cells were treated with ON for 1 h and their fluorescence intensity was determined. The results of this study showed that there was no significant difference in the amount of flourescence seen after the different periods of recovery compared to that in the IP-PL-untreated control (Fig. 8). This suggested that the pore formation during IP-PL treatment was transient and that minimal or no pore formation occured after the removal of IP-PL. Those results also indicated that the ON uptake enhancement required the presence of IP-PL and that the IP-PL treatment by itself did not cause an alteration in cellular permeability or integrity. These results are consistent with our earlier cellular uptake and cytotoxicity studies.

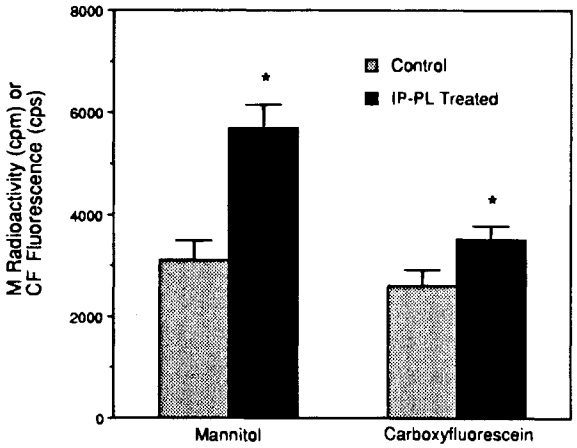

Fig. 7. Effect of IP-PL on Cellular Uptake of Carboxyfluorescein and Mannitol. Cells were incubated with ^{3}H mannitol (0.1 μCi/ml) or CF (1 μg/ml) in the presence of IP-PL (100 μM) at 37°C for 3 h. After incubation, the cells were washed and analyzed for fluorescence intensity or radioactivity. * indicates significant difference over controls ($p < 0.05$, n = 3).

DISCUSSION

In this study, we have demonstrated that the import peptide IP-PL can be used to provide enhanced cytoplasmic delivery of ON. The effect of IP-PL on ON uptake was found to be concentration- and cell type-dependent. The basis of the latter is not clear but is probably due to the difference in cellular interactions with the IP-PL. Cell type-dependent uptake of ONs was previously reported (12,13). The observed lower uptake of the ON:IP-PL complex in the phagocytic macrophage cell line compared to epithelial cell lines suggests that the uptake of the complex may not be mediated by the endocytic mechanism. Consistent with this observation, our cellular uptake studies using endocytic/metabolic inhibitors and low temperature support the non-endocytic uptake of ON mediated by the IP-PL. Our microscopic studies also showed a diffuse cytoplasmic distribution of the ON, further substantiating the non-endocytic uptake enhancement of IP-PL.

Once inside the cytoplasm, the ON appears to rapidly enter in the nucleus. At 10 min after treatment, intense nuclear fluorescence could be observed. This result suggests that the nuclear membrane does not present a significant barrier to the transport of ON. In agreement with this observation, Chin *et al.* (14) and Leonetti *et al.* (15) previously demonstrated that fluorochrome-labeled ONs, when injected intracellularly, rap-

idly entered the nucleus. The nuclear translocation process was completed within a few minutes after the injection. However, when ONs were given exogenously, they entered the nucleus at a much slower rate, i. e., <4% of the total cell-associated ON was in the nucleus after 1 h (12). The observed difference in the rate of nuclear uptake was believed to be due to the fact that exogenously administered ONs are taken up by cells via an endocytic process, and thus they are trapped in the endosomes. Our study which shows that ON when given as a complex with IP-PL can rapidly enter the nucleus suggests the non-endocytic uptake enhancement of the IP-PL.

The possibility that the IP-PL may enhance ON uptake by causing nonspecific cellular damage was investigated. This possibility, however, was ruled out since: a) our MTT studies showed that the cells remained viable and were able to proliferate normally following the IP-PL treatment; b) our reversibility studies indicated that cellular uptake of the free ON before and after IP-PL treatment was similar; and c) our uptake studies using M and CF showed that these compounds, despite of their smaller size, were taken up by cells to a much lesser extent than the larger ON.

Based on the data obtained and the known biological function of signal peptides, we propose that the IP-PL may promote the cellular entry of ON via a pore/channel formation. Until now, signal peptides have been known to facilitate transport of many secretoty proteins across eukaryotic and bacterial membranes through a hydrophilic protein-conducting channel. These peptides interact with lipid bilayers and adopt a structure that maximizes the clustering of its apolar residues and their penetration into the bilayer (9,10). The amphiphilic β-sheets that are folded into hollow barrel-like structures are thought to provide passive-diffusion pores through the membrane. Although this model appears to support our data, we did not provide direct evidence to show that the IP-PL peptide also acts in this manner. Further investigations into the interaction and conformation of this peptide in the lipid bilayer should increase our understanding of how the peptide might facilitate the ON uptake.

To be therapeutically effective, ONs must be able to cross cellular membranes and reach their targets intracellularly at a sufficiently high concentration. Although most existing methods of ON delivery have been shown to be effective in promoting the cellular uptake of ONs, they rely on the endocytic process. This mode of uptake, however, results in the ONs being trapped in endocytic vesicles and eventally degraded in lysosomes. Thus, eventhough their overall cellular uptake is increased, the actual amount of ONs reaching the target sites may still be low. In contrast, the import peptide carrier described here can promote the cellular uptake of ON via a non-endocytic process. Thus, this peptide has the potential to be used as an effective carrier for direct cytoplasmic delivery of ONs.

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