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Lipofectin-aided cell delivery of ribozyme targeted to human urokinase receptor mRNA

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Abstract A 37-mer hammerhead ribozyme has been designed to efficiently cleave the 1.4 kb mRNA of the urokinase plasminogen activator receptor (uPAR). Under in vitro conditions, the chemically synthesized ribozyme cleaved uPAR mRNA and inhibited its translation in a concentration-dependent fashion. The ribozymes were 5′-[^{35}S]thiophosphorylated and used as a model to analyze conditions for RNA delivery in a cultured human osteosarcoma cell system. Ribozymes degraded immediately in cell-conditioned medium but ribozymes complexed with lipofectin were protected from RNases for up to 22 h. Lipofectin rapidly transported ribozyme into the cell, where it accumulated almost exclusively in the cytoplasm. Thus, lipofectin dramatically enhances stability and cytoplasmic delivery of ribozymes, potentially enabling targeting of mRNA in vivo.

Key words: Hammerhead ribozyme; Delivery; Stability; Urokinase receptor; Lipofectin; HOS cell

1. Introduction

The urokinase receptor has been implicated in a series of normal and pathological processes of cell migration and invasion [1]. To evaluate the role of uPAR in such processes, catalytic RNA molecules targeted to cleave uPAR mRNA would be very valuable.

Experimental results demonstrate that complementary (antisense) RNA and DNA molecules can be used to interfere with the function of targeted RNA molecules (reviewed in [2]). Ribozymes, a new type of antisense RNA molecule capable of cleaving specific target RNAs, have recently been developed (reviewed in [3]). The cleavage process is mediated by a precise secondary structure formation between the catalytic and substrate RNA portions. The major advantage in using ribozymes is that they can cleave multiple target mRNA molecules, while the standard antisense molecules act only in equimolar ratio. For in vivo studies, however, utilization of pre-formed ribozymes is very limited: ribozymes, like other RNA molecules, are degraded immediately by RNases present in the culture medium.

Numerous studies have shown that cationic lipids, such as lipofectin [4], can effectively deliver RNA [5], DNA [6], or protein [7] molecules to mammalian cells in vitro or in vivo. The proposed mechanism of action involves lipofectin complex formation with RNA or DNA, and their subsequent fusion with the cell membrane [8]. Lipofectin has been shown to deliver pre-formed ribozymes to cells using serum-free culture conditions [9,10].

Here we provide evidence that ribozymes can be effectively introduced to cells cultured in serum-supplemented medium. Our results demonstrate that lipofectin can stabilize ribozymes in cell-conditioned medium presumably by making the ribozyme inaccessible to RNases. We show that ribozymes enter the cell very quickly and accumulate in the cytoplasm.

2. Materials and methods

Chemically synthesized 37-mer RNA (DNA Synthesis Facility, Wistar Institute) was 5′-phosphorylated with T4 polynucleotide kinase (Promega) to a specific activity of 1.9 Ci/mmol and 0.6 Ci/mmol using [γ-^{32}P]ATP and [γ-^{35}S]ATPS (Amersham) respectively, as described [11]. Target, 5′-capped uPAR mRNA (1370 nt) was synthesized from *Eco*RI (BRL)-linearized pT7TS-uPAR using an SP6 transcriptional system (MessageMachine, Ambion). Construction of pT7TS-uPAR was accomplished by subcloning uPAR coding sequences from pGEMuPAR [12] into pT7TS, which contained 5′ and 3′ untranslated regions of *Xenopus* β-globin mRNA (provided by Dr. P.A. Krieg, University of Texas, Austin).

Cleavage was performed by incubating the reaction mixture containing 0.05 μM mRNA, 1.0 μM ribozyme and 1 mM GTP in RNAzyme buffer (USB) at 50°C for 1 h. RNAs were separated on denaturing, 4% polyacrylamide/TBE gels, then electro-blotted to Nytran$^+$ membranes (Schleicher and Schuell) [13]. Northern analysis was performed according to standard protocols [14], using hybridization solution (3′Prime-5′Prime) and a cDNA probe specific to the 5′ end (nts 51–141) of uPAR mRNA (Accession: X51675). To generate this cDNA probe, we used a 5′ primer (5′GTCACCCGCCGCTGCTG3′) corresponding to nt 51–67, a 3′ primer (5′CCGTTGGTCTTACACTGCAT3′) corresponding to nt 141–122, and template pGEMuPAR [12] in a PCR reaction (Perkin-Elmer). The gel-purified 91 bp probe was labeled by random priming (Boehringer-Manheim). Signals on the Northern blot were quantitated using storage phosphor technology (PhosphorImager, Molecular Dynamics). The filters were also exposed to Kodak XAR film using an intensifier screen, at −70°C for 6 h.

uPAR protein synthesis was performed in a coupled transcriptional and translational system of rabbit reticulocyte lysate (TNT, Promega) using pGEMuPAR [12]. The incubation was performed in the presence or absence of ribozymes (0.25–25 μM) or antisense DNA (25 μM) for 90 min in [^{35}S]cysteine-supplemented lysate. Samples were separated by 10% SDS-PAGE. Fluorograms were generated as described [14].

To determine the stability of ribozymes in cells and culture medium, human osteosarcoma cells (HOS; ATCC) were seeded into 24-well plates (2×10^5 cells/well) 1 day prior to the experiment. Ribozyme was complexed with lipofectin (BRL) in a 2:5 ratio (w/w) by incubating at room temperature for 15 min as recommended. The ^{35}S-labeled ribozyme (0.6 μM) or its complex with lipofectin was added to cells and incubated at 37°C for 0.5, 2.5 and 22 h in 0.3 ml culture medium consisting of DMEM (Gibco) and 10% heat-inactivated fetal bovine

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serum (Gibco). At the end of incubation, the culture medium was collected and cells were harvested. RNA was extracted from cells and culture medium (30 μl) in the presence of 4 μg carrier tRNA (BRL) using RNazol (Biotecx) as recommended. After measuring radioactivity in aliquots of the aqueous and organic phases by scintillation counting, the RNA was precipitated, resolved in 80% deionized formamide (Clontech) and electrophoresed in a 16% PAGE/TBE gel under non-denaturing conditions. The dried gel was exposed to Kodak XAR film at −80°C for 1–7 days.

To determine the subcellular localization of ^{32}P-labeled ribozymes, nuclei and cytoplasm of the treated cells were separated as described [15]. Briefly, HOS cells (1×10^6) were cultured in 6-well plates in 1 ml of medium under the conditions described above. Cells were treated with the lipofectin-complexed ribozyme for 5, 10, 20, 30, 60 and 180 min, washed with PBS, collected in 0.5 ml trypsin-EDTA, transferred to a microtube, then lysed by vortexing in 150 μl buffer containing 0.5% NP40 in 10 mM Tris-HCl, pH 8.5, 1.5 mM $MgCl_2$ and 140 mM NaCl, for 2 min at 25°C. The extent of cell lysis was assessed by examining an aliquot of the cell lysate by microscopy. Nuclei and cytoplasm were separated by centrifuging the lysate at 12,000 rpm in a microfuge. RNAs were extracted from the pelleted nuclei, from the supernatant cytoplasm, and from an aliquot (30 μl) of culture medium with RNazol. Samples were analyzed in a 16% PAGE/TBE gel under denaturing (8 M urea) conditions.

Secondary structure predictions for RNAs were generated by the MacDNASIS program (Hitachi) using a minimum free energy folding algorithm [16].

3. Result and discussion

3.1. Optimizing ribozyme design

Based on the sequence requirements for hammerhead structure formation between target RNA and ribozyme [17], we have designed ribozymes targeted to cleave uPAR mRNA. First, we analyzed all of the 34 potential ribozyme cleavage sites (GUN, where n = G,U,A,C) on the uPAR mRNA. We have selected sites on the mRNA that were predicted not to be involved in stable structure formation with the neighboring sequences, because the secondary structure of the target RNA can inhibit assembly of the hammerhead domain [18,19]. As a further evaluation, we predicted secondary structures for all of the ribozymes that could be targeted to the selected sites. The ribozyme, shown with the complementary sequences of uPAR mRNA (nt 124–139) (Accession: X51675), which was one of those predicted to fold into the desired secondary structure (Fig. 1), was chosen for further studies.

3.2. Cleavage of uPAR mRNA by ribozyme

The cleavage reaction was performed using 20-fold molar excess of the chemically synthesized 37-mer ribozyme over the in vitro transcribed 1370 nt uPAR mRNA substrate. The reaction mixture, separated by denaturing PAGE, was analyzed by Northern blot using a radiolabeled probe specific for the 5′ end

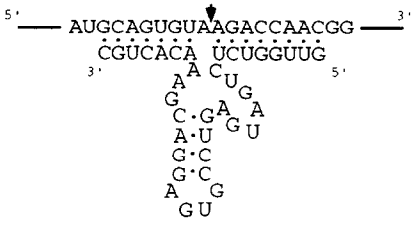

Fig. 1. Predicted secondary structure of the ribozyme with the uPAR mRNA template. The cleavage site is indicated by an arrow.

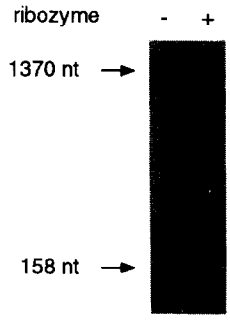

Fig. 2. Specific cleavage of uPAR mRNA by ribozyme. The target 1370 nt uPAR mRNA was incubated with or without ribozyme for 1 h at 50°C. Samples were separated by denaturing 4% PAGE and transferred to a filter. The autoradiogram of the Northern blot, probed with a cDNA corresponding to the 5′ end of uPAR mRNA, is shown. The positions of the visualized 1370 nt substrate mRNA and the 158 nt 5′ cleavage product are indicated.

of uPAR mRNA. This probe selectively identifies the 5′ cleavage products with the predicted size of 158 nt and the substrate mRNA (1370 nt) but can not hybridize to the 3′ cleavage product (1212 nt) (Fig. 2). Phosphor image analysis of the Northern blot shown in Fig. 2 demonstrated that 3.9% of the substrate uPAR mRNA was specifically cleaved by the ribozyme. This result clearly demonstrates that the ribozyme is an enzymatically functional molecule. The ribozyme activity was also tested in rabbit reticulocyte lysates. Using the coupled TNT system, we transcribed and translated the uPAR mRNA in the same lysate. The TNT system made isolation of the mRNA unnecessary and made it more likely that the mRNA secondary structure would stay in its nascent form. Ribozyme specifically inhibited uPAR protein synthesis in a concentration-dependent manner (Fig. 3). Synthesis of the ~50 kDa and ~70 kDa unspecified proteins from internal mRNAs present in the reticulocyte lysates was not inhibited. The conventional antisense oligonucleotide that was targeted against the identical mRNA sequences was only marginally effective (Fig. 3). These data suggest that ribozyme might be active under physiologically more relevant cell lysate conditions.

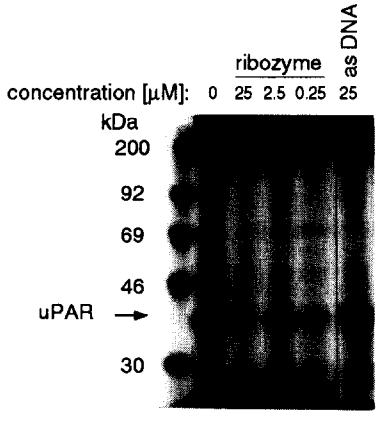

Fig. 3. Inhibition of uPAR synthesis by ribozyme. Coupled transcription and translation of uPAR was performed in the presence of ribozyme or antisense DNA. Samples were incubated in [^{35}S]cysteine-supplemented rabbit reticulocyte lysates at 37°C for 90 min, then separated by 10% SDS-PAGE. The position of the 37 kDa human uPAR is indicated on the fluorogram.

3.3. Stability of ribozyme in HOS cells and in cell culture medium

To investigate the effect of lipofectin on the stability and cellular uptake of the ribozyme, we have introduced the phosphatase-resistant [^{35}S]thiomonophosphate label [20] to the 5′ end of the synthetic ribozyme. Considering that RNA is degraded predominantly by 3′ exonucleases present in the serum and cell supernatants [21], the 5′-end labeling should not increase the ribozyme overall stability. We used non-denaturing TBE-PAGE and fluorography to detect ribozyme which was recovered from the culture medium and from the treated cells. Ribozymes directly added to the culture medium of HOS cells were degraded immediately, presumably by RNases present in the serum (Fig. 4, lanes 1–3). However, ribozymes complexed with lipofectin prior to the experiments survived and were still detectable after a 22 h incubation period (Fig. 4, lanes 4–6). These results suggest that lipofectin confers RNase resistance to ribozymes in cell culture medium possibly by steric inhibition.

Analysis of RNA isolated from cells that were treated with ribozyme alone, as expected, resulted in no detectable amount of intact ribozyme (Fig. 4, lanes 7–9). However, ribozyme could be recovered from cells that had been treated with the lipofectin–ribozyme complex (Fig. 4, lanes 10–12). Intact ribozymes were detectable even at the end of a 22 h incubation period. About 1–3% (~$2–6 \times 10^{-12}$ mol) of the added 300 μl 0.6 μM ribozyme could be recovered from HOS cells (2.5×10^5) treated for 0.5 h, suggesting that lipofectin can efficiently transfer ribozymes to the cells, where the ribozyme accumulates at ~$1–10 \times 10^6$ molecules/cell.

3.4. Subcellular distribution of the lipofectin delivered ribozyme

We have previously reported that DNA entrapped in phospholipid liposomes were successfully delivered into cells in culture [22]. The transport was fast (~5–10 min) and about 90% of the delivered DNA accumulated in the nuclei within 20 min. To evaluate the subcellular localization of the lipofectin-delivered ribozymes, we 5′-end labeled the ribozymes with the more easily detectable [^{32}P]monophosphate. Cells treated with ribozyme–lipofectin complex were separated to cytoplasmic and nuclear fractions after different incubation periods. RNAs isolated

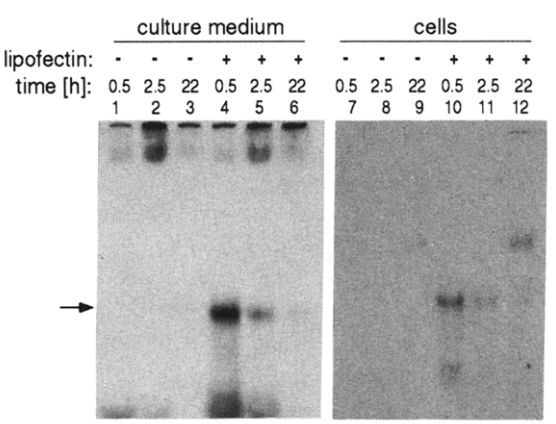

Fig. 4. Ribozyme stability in culture medium and cells. HOS cells were incubated with ^{35}S-labeled ribozyme which was or was not reacted to complex with lipofectin. RNAs isolated from the medium and cells were separated by non-denaturing 16% PAGE. Position of the intact ribozyme is indicated on the fluorograms by an arrow.

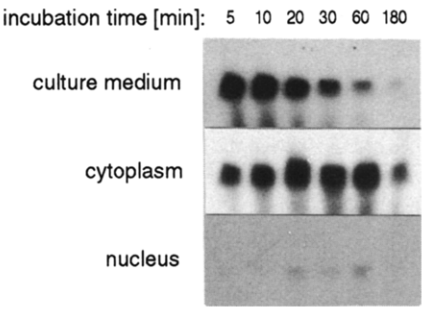

Fig. 5. Subcellular localization of the lipofectin delivered ribozyme. HOS cells were incubated with lipofectin-complexed ^{32}P-labeled ribozyme. RNAs were extracted from the culture medium, and from isolated nuclei and cytoplasm. Samples were separated by denaturing 16% PAGE. The autoradiogram of the dried gel is shown.

from the cytoplasm, nuclei and culture medium were analyzed. The results presented in Fig. 5 demonstrate that ribozyme was taken up very quickly by the HOS cells, and the majority of intact ribozymes were located in the cytoplasm. After a 22 h incubation period, no ribozyme could be detected in any of the analyzed fractions (not shown). Presumably, serum and cellular enzymes removed the monophosphate labeling, which, unlike the 5′ thiomonophosphates, is susceptible to phosphatases [20]. These data suggest the superiority of ^{35}S over ^{32}P for labeling the 5′ end of the pre-formed ribozymes that are planned for use in cellular studies. Reports have already suggested that lipofectin-complexed molecules are preferentially transported to the cytoplasm [23], while others proposed nuclear localization of the delivered products [9]. One possible explanation for the discrepancies could be the different methods for isolation of the nuclear fraction. To achieve a better separation of the subcellular fractions we used a more stringent nuclei isolation procedure, which was specifically recommended for extranuclear RNA analysis [15].

In summary, these studies suggest that positions 124–139 of the 1.4 kb uPAR mRNA are accessible for ribozyme-mediated cleavage. Our data indicate that lipofectin, in addition to assisting ribozyme delivery to cells, also protects the ribozyme from degradation by serum nucleases. These results suggest that preformed ribozyme delivered with lipofectin may be useful in the analysis of the role of uPAR in different biological processes.

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