Visualizing the dynamics of viral replication in living cells via Tat peptide delivery of nuclease-resistant molecular beacons

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In this study, we describe the use of nuclease-resistant molecular beacons (MBs) for the real-time detection of coxsackievirus B6 replication in living Buffalo green monkey kidney (BGMK) cells via Tat peptide delivery. A nuclease-resistant MB containing 2'-O-methyl RNA bases with phosphorothioate internucleotide linkages was designed to specifically target an 18-bp 5' noncoding region of the viral genome. For intracellular delivery, a cell-penetrating Tat peptide was conjugated to the MB by using a thiol–maleimide linkage. Presence of the Tat peptide enabled nearly 100% intracellular delivery within 15 min. When the conjugate was introduced into BGMK cell monolayers infected with coxsackievirus B6, a discernible fluorescence was observed at 30 min after infection, and as few as 1 infectious viral particle could be detected within 2 h. The stability and the intracellular delivery properties of the modified MBs enabled real-time monitoring of the cell-to-cell spreading of viral infection. These results suggest that the Tat-modified, nuclease-resistant MBs may be powerful tools for improving our understanding of the dynamic behavior of viral replication and for therapeutic studies of antiviral treatments.

Results and Discussion

Design and Characterization of Nuclease-Resistant MB. An MB (MB CVB6-Tat) targeting an 18-bp region of the 5' untranslated region of CVB6 was designed. The DNA backbone was modified with sulfur-substituted 2'-O-methyl oligoribonucleotides for improved nuclease resistance (17–20). A Tat peptide for intracellular delivery was conjugated to the thiol group at the quencher end by using a maleimide group placed at the N-terminus of the peptide (Fig. 1L) (21, 22). As expected, the modified MBs were highly resistant to nuclease cleavage by DNase I (Fig. 1B). In contrast, an unmodified MB was susceptible to nuclease degradation, resulting in almost instantaneous increase in fluorescence. The dual modifications had no effect on the hybridization kinetics of the MB, as a rapid increase in fluorescence was observed in the presence of a complementary target (Fig. 1C).

Intracellular Delivery of Tat-Modified MB CVB6. The intracellular delivery efficiency was tested by incubating 0.5, 1, or 2 μM MB–target hybrids with a monolayer of Buffalo green monkey kidney (BGMK) cells. Fluorescence was detectable in 100% of the living cells as early as 15 min after introduction of the MB–target hybrids (Fig. 24). The time-lapse images showed that the cellular uptake increased after 15 min and reached saturation after 1 h of incubation. The fluorescence intensity was constant for up to 12 h, indicating that the MB–target hybrids were retained inside the cells after delivery and remained resistant to the intracellular RNase H.


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Supporting Information online.

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Unhybridized MB was also introduced into BGMK cells, and no fluorescence was detected during the same 12-h period, again indicating the intracellular resistance of the modified MBs to nuclease attack (Fig. 2B). In contrast, in the absence of Tat, no internalization of MBs was observed, and fluorescence was detected only in the medium (Fig. 2B), confirming the effectiveness of the Tat peptide for rapid intracellular delivery.

Detection of CVB6 Infection by Tat-Modified MBs. After validating the properties of Tat-modified MBs, their ability to detect viral RNA was tested. A confluent monolayer of BGMK cells was first incubated with 1 μM MB for 30 min before being infected with 10-fold serial dilutions of CVB6, followed by fluorescence microscopy. Compared with uninfected cultures (0 pfu), where no fluorescent cells were present independent of time, a greater number of fluorescent cells were detected at 2 h post infection (p.i.) for the culture infected with a viral dosage corresponding to 1 pfu (Fig. 3A). The higher number of fluorescent cells compared with plaque-forming units is likely due to the fact that not all viruses that infect a cell are necessarily able to complete the replication cycle (23). This result is also consistent with the higher infectious virus titers observed by using the quantal assay, which is based on the direct microscopic viewing of cells for virus-induced cytopathic effects, rather than the plaque assay (24). This 2-h detection window is substantially faster than a similar approach reported using cell fixation/permeabilization (16). It is possible that the rapid and noninvasive intracellular delivery enables hybridization with viral RNA to occur shortly after virus uncoating without the possibility

Fig. 1. MB backbone modification and nuclease sensitivity study. (A) A schematic representation of the Tat-modified, nuclease-resistant MB. The phosphodiester bond was modified by replacing a nonbridging oxygen with sulfur and the 2′-sugar deoxy with 2′-O-methyl group. At room temperature, the thiol group at the quencher end reacted (≈2 h) with a maleimide group placed at the N terminus of the peptide to yield a chemically stable thioether bond. (B) Nuclease sensitivity assays using ribonuclease-free DNase I. The fluorescence of the nuclease-resistant MB is shown in yellow, and the fluorescence of an unmodified MB is shown in red. The background fluorescent signals (shown in black and green) without DNase I addition are also shown. (C) Kinetics of hybridization of Tat-modified MB CVB6 with (green) or without (orange) complementary oligonucleotides.

Fig. 2. Intracellular delivery of MB CVB6-Tat–target hybrids (A) or MB without Tat modification or without targets (B). BGMK cells were incubated with 1 μM MB for 12 h, and images were captured by using a fluorescent microscope. (Scale bar, 20 μm.)
of degradation caused by fixation/permeabilization. To our knowledge, detection of 1 pfu of CVB6, or any other enterovirus, at 2 h p.i. has never been reported.

Using the 2-h infection window, we tested the utility of Tat-modified MBs to quantify infectious CVB6 dosages. Cells were infected with 1–200 pfu of CVB6 per well, and the average number of fluorescent cells was recorded. A linear correlation was obtained by plotting the number of fluorescent cells versus plaque-forming units (Fig. 3B). The number of plaque-forming units can be determined easily by using the correlation obtained after direct counting of fluorescent cells. More importantly, the method can be used to provide rapid quantification of infectious CVB6 dosages within 2 h p.i. compared with the minimum 48-h incubation period for the plaque assay.

Real-Time Monitoring of Cell-to-Cell Spreading of CVB6. The ability to detect infected cells continuously should allow one to follow the spreading of infectious viruses on a real-time basis. To determine whether this was indeed possible, BGMK cells were infected at a very low infection dosage (multiplicity of infection: 0.01 pfu/cell) and monitored continuously by using a fluorescence microscope in a fixed area for 12 h. Fig. 4 shows the cell-to-cell progression of virus spreading at 6 representative time points [A real-time movie of virus spreading is provided in supporting information (SI) Movie S1]. Several infected cells were observed at 15 min p.i., suggesting that the viruses entered the cells and started the uncoating process within 15 min. The number of fluorescent cells slowly increased with time, suggesting continuous virus infection. By 6 h p.i., a further outward spread of fluorescent cells was observed, indicating the secondary spreading of infection from progeny virions to cells surrounding the initial infected cells. The number of fluorescent cells continued to increase with time, and infection spread outward to the entire observation area by 12 h p.i. The majority of infected cells remained adherent, and some fluorescence was visible outside the cells, indicating that the fluorescent hybrids with viral RNA entered the extracellular region as a result of the release of progeny virions during cell lysis.

In summary, we demonstrated the use of nuclease-resistant, Tat-modified MB for real-time monitoring of viral replication and infection. This method is simple because it requires no cell pretreatment (e.g., fixation/permeabilization) and can be used to study cell-to-cell viral spreading. This method is particularly attractive when applied to viruses with very slow growth, nonlytic viruses, and those that do not produce detectable cytopathic effects in infected cells. Compared with conventional viral plaque assays, which detect infection based on cell lysis only and may take days to weeks to complete, this real-time approach provides an opportunity to study the progress of the entire infectious cycle.

Materials and Methods

BGMK Cell Culture. BGMK cells obtained from American Type Culture Collection (passages 50–60) were grown in 400 mL of 1/11003 autoclavable minimum essential medium (AMEM; Irvine Scientific) containing 4 mL of 7.5% NaHCO3, 8 units/mL penicillin, 1,000 units/mL streptomycin, 2 mg/mL kanamycin, 2,000 units/mL nystatin, 80 mM L-glutamine), and 40 mL of FBS (Sigma–Aldrich) at 37°C in a 5% CO2 atmosphere. PBS [1/11003 PBS (0.01 M phosphate (pH 7.4), 0.138 M NaCl, and 2.7 mM KCl) and Tris-buffered saline solution [1/11003 TBSS (0.05 M Tris (pH 7.4), 0.28 M NaCl, 10 mM KCl, and 0.82 mM Na2HPO4] were used for washing steps in the plaque assay and MB analysis, respectively.

Virus Preparation. Virus stocks of CVB6 Schmitt strain (ATCC VR-155) were allowed to proliferate on BGMK cells for 2 days at 37°C in a 5% CO2 atmosphere and collected by freeze–thawing (3 times) infected flasks demonstrating >80% lysis and extracting the cell lysate with chloroform. The CVB6 virus stock was stored at −80°C.

Fig. 3. In vivo detection of CVB6 in BGMK cells. (A) Visualization of BGMK cells infected with 0, 1, or 105 pfu at 2 h p.i. (B) The correlation between the number of plaque-forming units and fluorescent cells at 2 h p.i. Error bars represent the standard deviation of 3 replicate experiments. (Scale bar, 40 µm.)

Fig. 4. Real-time detection of viral spreading. BGMK cells were first incubated with 1 µM MB, infected with CVB6 at an multiplicity of infection of 0.01 pfu/cell, and monitored by using a fluorescent microscope. (Scale bar, 20 µm.)
Plaque Assay. The CVB6 virus stock was thawed, and then a series of 10-fold serial dilutions in 1× PBS were prepared. Confluent, 1-day-old BGMK cell monolayers in 12-well, 22.1-mm dishes (Costar, Corning) were infected with 1 mL of virus dilution. After 90 min of adsorption at room temperature, the solutions were aspirated, and 1 mL of 100 mL of 2% carboxymethylcellulose (CMC) sodium salt (Sigma–Aldrich) containing 100 mL of 2 AMEM (Irvine Scientific) with 2 mL of 7.5% NaHCO3, 4 mL of 1M Heps, 2 mL of NEAA, 5 mL of A/B-L, and 4 mL of FBS (Sigma–Aldrich) was added into each well. After 2 days of incubation at room temperature, the CMC overlay was removed, and the cells were treated with 0.8% crystal violet/3.7% formaldehyde solution overnight. Excess stain was removed by washing with de-ionized water and the virus plaques were counted.

Design of Nuclease-Resistant MB. MB CVB6 was designed on the basis of an alignment of the sequences of the enterovirus strains obtained from Genbank database. The DNA folding program mfold (www.bioinfo.riu.edu) and IDT SciTools (www.idtdna.com/SciTools/SciTools.aspx) were used to predict the thermodynamic properties and the secondary structures of MBs. CVB6 5′-6-FAM (fluorescein)-GCCGGCTGACTTACGGCGGAGGGCGAGGC-hiol-dG-GGC-13′ [stem sequence is underlined; DABCYL = 4-(4-dimethylaminophenylazo)benzoic acid] possessing a 2′-O-methylribonucleotide backbone with phosphorothioate internucleotide linkages was synthesized (TIB Molbiol) to be specifically hybridized to the untranslated region of the enterovirus genome. The thiol group at the 5′ hydroxyl end is a reactive group attached to the N terminus of the Tet peptide to form a thiol–maleimide bridge. MB CVB6 was suspended in 100 mM Tris/HCl (pH 8.0) buffer containing 1 mM MgCl2 to make the concentration 100 μM for the subsequent studies.

DNase Sensitivity. To test the nuclease sensitivity of MB CVB6, the fluorescence of a 500-μM solution of 1 μM MB CVB6 was recorded as a function of time at room temperature (aspirated-free MB dilute in PBS for 1×). The fluorescence signals (excitation 495 nm, emission 521 nm) were measured for 30 min by using an RF-S51 spectrofluorometric detector (Shimadzu). As a comparison, the fluorescence of a 500-μM solution of 1 μM MB possessing deoxyuribonucleotide backbone 5′-6-FAM -GCCGGCTGACTTACGGCGGAGGGCGAGGC-DABCYL-13′ (MB CVB1), stem sequence is underlined was recorded after adding 5 units of ribonuclease-free DNasel. Without adding ribonuclease-free DNase 1, the background fluorescent signals of a 500-μM solution containing 1 μM MB CVB6 or MB CVB1 were monitored for 30 min.

Peptide Conjugation. One hundred and fifty monoclonal N-terminal maleimide-modified Tat peptide H-Tyr-Gly-Arg-Lys-Arg-Gln-Arg-Arg-NH2-C2H2-N-imaleimide (Global Peptide) was mixed with 100 μM thiolated MB in the dark for 2 h to form a stable thiol–maleimide linkage. The peptide-linked MB complex was dialyzed overnight in Slide-A-Lyzer Mini Dialysis Units, 10,000 molecular weight cutoff, to remove the unconjugated peptide/MBs (Pierce). The peptide-conjugated MB CVB6-Tat was stored at −20°C until used for experiments.

Cellular Delivery of Peptide-Conjugated MBs. BGMK cells were seeded into the 8-well Lab-Tek Chambered Coverglass (Fishier Scientific) at 37°C in 5% CO2 in air and cultured to >90% confluence. After removal of the incubation medium, the cell monolayer was washed twice with 1× TBSS and then treated with an efficiency of Tat peptide-mediated intracellular delivery, nonconjugated MB CVB6 or MB CVB6-Tat was mixed with complementary oligonucleotides (5′-CTCGGCCCTGAAATGGC-3′) to the loop region at an MB/oligonucleotide molar ratio of 1:1. BGMK cells were incubated at 37°C in the dark with 1× Leibovitz L-15 medium (Invitrogen) containing either preformed nonconjugated MB CVB6 hybrids or MB CVB6-Tat hybrids at MB concentrations of 0.5, 1, or 2 μM. The Leibovitz L-15 contains no phenol red to eliminate autofluorescence and increase optical transmission. To record the image, the chamber well was placed on the Zeiss Axiosvert 40 CFL inverted fluorescence microscope stage and was marked to permit the repeated observation of the chosen region in the cell monolayer. As soon as the positive fluorescent signals were observed inside the cells, the chamber well was kept on the microscope stage instead of returning it to the 37°C incubator. All assays were carried out over a period of 12 h, and the fluorescence images were taken at intervals of 5 min.

Progression of Viral Infection in Living Cells. BGMK cells were cultured to >90% confluence in the 8-well Lab-Tek Chambered Coverglass (Fishier Scientific) at 37°C in 5% CO2 atmosphere. After incubation for predetermined time periods, the slides were removed from the 37°C incubator, and the growth medium was aspirated. Following 2 washes with 1× TBSS, the cells were incubated with 1 μM MB CVB6-Tat for 15 min at 37°C in the dark for 30 min. Without washing away the incubation medium, the chamber wells were oriented on the microscope stage; the cells were infected with 10-fold virus dilutions in 1× Leibovitz L-15 medium and were observed under the Zeiss Axiosvert 40 CFL inverted fluorescence microscope at room temperature for 12 h. The fluorescence images were recorded at intervals of 5 min.

Fluorescence Microscopy and Image Processing. Living cell imaging was performed on a Zeiss Axiosvert 40 CFL inverted microscope equipped with a 12-V, 35-W halogen lamp (for the phase-contrast images) and an HBO 50W/A mercury lamp (for the fluorescence images). The objectives used were a 5×/0.12. A-Plan, a 10×/0.25 A-Plan, a 20×/0.50 EC Plan-NEOFLUAR, and a 40×/0.50 LD A-Plan (Zeiss). 6-FAM-labeled fluorescent hybrids were detected by using a filter set consisting of a D480/30-nm exciter, a D535/40-nm emitter, and a 505-dichroic long pass barrier. Chroma Technologies were equipped with an ProgRes TM+Monochrome CCD camera (Jenoptik). Both phase-contrast and fluorescence images were analyzed by using Image-Pro PLUS analysis software (Media Cybernetics). All settings for image processing were kept constant, and the exposure time for image capture was adjusted, if necessary, to maintain output levels similar to those observed under the fluorescence microscope.

Enumeration of Fluorescent Cells. To calculate the infected cells (fluorescent cells) in each chamber well, 39 fields within the well were randomly chosen, and the fluorescence images were collected at 10× magnification. The number of fluorescent cells within the area was counted by Image-Pro PLUS analysis software.

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