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Inhibition of influenza virus RNA (PB2 mRNA) expression by a modified DNA enzyme

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ABSTRACT

DNA enzymes are RNA-cleaving single stranded DNA molecules. The structure and the catalytic domain of a DNA enzyme were determined by Santro *et al.* in 1997. In this study, we have designed several types of DNA enzymes (PB2Dz) targeted to the PB2 mRNA translation initiation region of influenza A virus, and examined their cleavage kinetics, nuclease resistance, and a luciferase gene reporter assay. Using a synthetic substrate, these DNA enzymes were shown to have cleavage activity that is dependent on the length of the substrate recognition domain. To confer serum nuclease resistance to the DNA enzymes, we designed a new type of DNA enzyme that has the N3'-P5' phosphoramidate modification (PB2Dz-N) at each terminal. We examined the activity of this DNA enzyme in vivo. The DNA enzymes used in this study inhibited the expression of the PB2-luciferase gene in COS cells. These results suggest that DNA enzymes are potentially useful as gene inactivating agents of influenza A virus.

INTRODUCTION

Influenza A viruses can cause acute respiratory disease in humans and animals. The virus is a negative stranded RNA virus with an eight segmented genome encoding 10 different structural and non-structural proteins. The viral RNA polymerase is encoded by the *PB2*, *PB1*, and *PA* genes. The *PB2* and *PB1* gene products function cooperatively in cap-dependent mRNA transcription. PB2 provides both mRNA cap recognition and cap cleavage activities (1).

Antisense and ribozyme technologies are major tools in gene inactivation approaches for human gene therapy (2). These oligonucleotides can block messenger RNA translation through sequence-specific hybridization or cleavage. We previously tested the ability of antisense phosphodiester and phosphorothioate oligonucleotides to inhibit the expression of the influenza virus PB2 genes in cultured cells and mice (3,4).

Recently, novel catalytic molecules made of single stranded DNA (DNA enzymes) were obtained through *in vitro* selection (5). One model of a DNA enzyme (motif 10-23 nt) comprises a catalytic domain of 15 nt, flanked by two substrate recognition domains, and binds and cleaves any single stranded RNA between an unpaired purine and a paired pyrimidine. In another study, this DNA enzyme was shown to be potentially useful for clinical applications as a purging tool, with less potential for disrupting normal gene expression (6-8).

In this study, we designed a DNA enzyme (PB2Dz) targeted to the PB2 mRNA translation initiation region of influenza A virus (A/PR/8/34), which has the N3'-P5' phosphoramidate modification (PB2Dz-N) at each terminus to confer nuclease resistance in serum (Figure 1). We examined the kinetics and the inhibitory effects in mammalian cells using reporter constructs with a luciferase gene. These results suggest that the DNA enzyme is a potentially useful agent for the inhibition of influenza A virus replication.

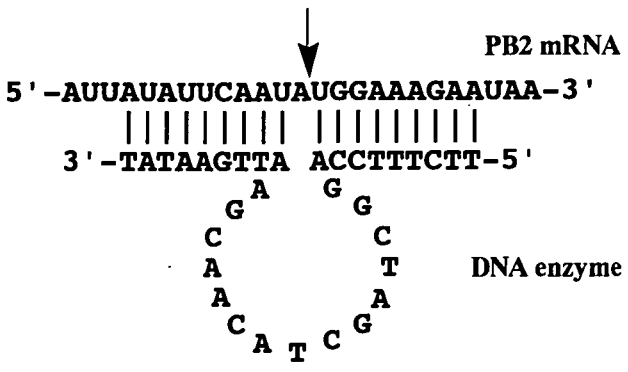

Figure 1. Structure of the DNA enzyme used in this study

MATERIALS AND METHODS

The efficacy of the DNA enzyme *in vitro* was determined by measuring the rate of RNA cleavage under multiple turnover conditions. Reactions were performed with 0.02 μM DNA enzyme and five different concentrations of an FITC-labeled synthetic RNA substrate (0.2, 0.4, 1.0, 1.6, or 3.2 μM) with 25 mM $MgCl_2$ in 50 mM Tris-HCl[pH 7.5] and were incubated for 5, 10, 20, 30, or 60 min. The reactions were stopped with 50 mM EDTA, 9M urea, and 0.1% xylene cyanol, denatured for 1 min at 95℃, and cooled on ice. The cleavage product fragments and the unreacted substrate in these samples were separated by electrophoresis on an 18% polyacrylamide gel. The extent of the reaction at each substrate concentration and time point was determined by the density of the gel image produced through FLA-2000G (Fuji Film, Japan). Kinetics of DNA enzymes were obtained from the slope of the curves that represented the time course of each reaction, and k_{cat} and K_M were calculated from an Eadie-Hofstee plot.

The efficacy of the DNA enzyme in mammalian cells was determined by a luciferase assay. COS cells were cultured RPMI-1640 medium (SIGMA) supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37℃ in 5% CO_2. In the luciferase assay, COS cells were plated at 2×10^5 cells/well in a 6-well plate and were cultured for 24 h. Each DNA enzyme (final concentration 0.3 μM) and 1 μg of target expressing plasmid vector (pCEP4/Luc/PB2) were co-transfected with the FuGENE™6 transfection reagent (Roche Diagnostics K.K.) in 100 μl of RPMI-1640 medium. After 24 h, the COS cells were harvested and the luciferase activity was measured with a PicaGene kit (Toyo-inki).

Table 1. Kinetics of PB2 mRNA cleaving DNA enzymes

DNA enzyme	PB2Dz	PB2Dz-N
$k_{cat}(min^{-1})$	9.1×10^{-3}	1.6×10^{-2}
$k_{cat}(min^{-1})$	6.8×10^{-1}	6.0×10^{-1}
$k_{cat}/K_M(\mu M^{-1}min^{-1})$	1.3×10^{-2}	2.7×10^{-2}

Rate constants were measured with 25 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.5) at 37℃. (multiple turnover conditions)

RESULTS AND DISCUSSION

Multiple turnover kinetics were used to examine the efficiency of DNA enzymes *in vitro*. Unmodified PB2Dz and N3'-P5' phosphoramidate modified PB2Dz-N were incubated with an excess of the FITC-labeled synthetic RNA. The kinetic parameters, k_{cat} and K_M, were determined from an Eadie-Hofstee plot (Table 1). The relative efficiency of PB2Dz was increased 2-fold by the presence of the N3'-P5' phosphoramidate modification on the DNA enzyme, in terms of the value of k_{cat}/K_M. Therefore, these results suggest that this modification does not influence the kinetics of PB2 mRNA cleavage.

To evaluate the intracellular activities of DNA enzymes, we co-transfected each DNA enzyme and a plasmid vector (pCEp4/Luc/PB2), which encoded a chimeric target *PB2* sequence and a gene for luciferase, with the FuGENE™6 transfection reagent in COS cells. When the DNA enzymes were transfected in COS cells, the luciferase activity decreased (data not shown). These results indicate that the DNA enzyme had highly inhibitory effects in mammalian cells.

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