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An efficient screening system for influenza virus cap-dependent endonuclease inhibitors

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The synthesis of influenza virus mRNA is primed by capped (m^7GpppNm-) short RNAs that are cleaved from RNA polymerase II transcripts by a virally encoded endonuclease. This cap-dependent endonuclease activity called "cap-snatching" may provide a unique target for novel anti-viral agents. To screen candidate inhibitors, it is essential to establish a method for producing efficiently a capped RNA substrate and a convenient assay for the cap-snatching activity. A 3′-biotinylated short RNA was prepared by *in vitro* transcription, purified by C_{18} reverse-phase column chromatography, and subjected to a capping reaction involving three recombinant capping enzymes. This capped RNA was shown to be an efficient substrate for the cap-snatching assay. Cap-snatching activity was then measured with the novel pull-down assay developed in this study, which is based on the streptavidin–biotin interaction. A known inhibitor for the cap-snatching reaction was evaluated by the pull-down assay, demonstrating the efficacy of the established screening system.

1. Introduction

Influenza virus causes acute respiratory infections in humans that sometimes progress to severe pulmonary diseases, resulting in considerable mortality among elderly and other high-risk individuals. Because the currently available vaccines do not protect completely against influenza virus infections, there is an urgent need for the development of anti-influenza therapeutic agents. There are currently only two kinds of licensed drugs for influenza virus, neuraminidase inhibitors (oseltamivir and zanamivir) and M2 channel blockers (amantadine and rimantadine) (De Clercq, 2006; Salomon and Webster, 2009; Das et al., 2010; Das, 2012). However, many influenza type A virus strains have become resistant to these M2 channel blockers and/or neuraminidase inhibitors (Hayden et al., 1989; Bright et al., 2005; Le et al., 2005).

The influenza virus genome consists of eight segmented RNA molecules with negative polarity. The single-stranded genomic RNAs are transcribed and replicated by a virally encoded RNA-dependent RNA polymerase, which is composed of three subunits, PB1, PB2, and PA (Horisberger, 1980). The translation of the viral mRNAs by host ribosomes requires that the mRNAs are capped, to allow their recognition and binding. However, influenza virus RNA polymerase does not carry the enzymatic activities required to add a 5′ cap to its own mRNAs, so the necessary cap structure is "snatched" from host pre-mRNA transcripts (Bouloy et al., 1978, 1980; Plotch et al., 1981; Ulmanen et al., 1981). Influenza virus RNA polymerase binds to the host pre-mRNA transcripts through their cap structures and cleaves them to generate capped RNAs of 11–13 nucleotides, which are used as the primers for viral transcription (Bouloy et al., 1978, 1980; Plotch et al., 1981; Ulmanen et al., 1981). This cap-snatching reaction may be used as a novel target for the development of anti-influenza-virus agents because this reaction is a unique and essential mechanism for influenza virus transcription.

In eukaryotes, most cellular and viral mRNAs have 5′ cap structures consisting of 7-methylguanosine linked to the initiating nucleotide of the transcripts *via* an inverted 5′–5′ triphosphate bridge (m^7G(5′)ppp(5′)N; Cap 0) (Banerjee, 1980; Mizumoto and Kaziro, 1987; Furuichi and Shatkin, 2000; Decroly et al., 2011). The Cap 0 structure is essential for the growth of *Saccharomyces cerevisiae* (Shibagaki et al., 1992; Mao et al., 1995) and the survival of mammalian cells (Yue et al., 1997). The Cap 0 structure is further methylated at 2′-*O*-ribose, either at the first nucleotide (m^7G(5′)ppp(5′)Nm; Cap 1) or at the first and second nucleotides (m^7G(5′)ppp(5′)NmNm; Cap 2) in higher eukaryotes by nucleoside

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2′-*O*-methyltransferases (Banerjee, 1980; Furuichi and Shatkin, 2000).

The Cap 0 structure is formed by three enzymatic reactions: (1) RNA 5′-triphosphatase (TPase) hydrolyzes the 5′ triphosphate end of the pre-mRNA to a diphosphate; (2) mRNA guanylyltransferase (GTase) adds a guanosine monophosphate (GMP) residue to the diphosphate end of the RNA; and (3) m^7G-methyltransferase (m^7G-MTase) methylates GpppN- to form the Cap 0 structure (Banerjee, 1980; Mizumoto and Kaziro, 1987; Furuichi and Shatkin, 2000; Decroly et al., 2011). These three enzymes are present in the virion of vaccinia virus, reovirus, and cytoplasmic polyhedrosis virus (Shuman et al., 1980). Vaccinia virus also uses cap1 mRNA nucleoside 2′-*O*-methyltransferase (cap1 MTase) to convert Cap 0 to the Cap 1 structure (Schnierle et al., 1992). Many cellular capping enzymes and m^7G-MTase have also been isolated from humans (hCap1, hMet1) (Tsukamoto et al., 1998a,b; Yamada-Okabe et al., 1998), mice (Mce1) (McCracken et al., 1997), *Caenorhabditis elegans* (CEL-1) (Takagi et al., 1997), *S. cerevisiae* (CEG1, CET1, ABD1) (Shibagaki et al., 1992; Mao et al., 1995; Tsukamoto et al., 1997), and *Schizosaccharomyces pombe* (Pce1, Pct1) (Shuman et al., 1994; Pei et al., 2001). Recently, human cap1 mRNA (nucleoside-2′-*O*-)-methyltransferase (hMTr1) (Belanger et al., 2010) and cap2 mRNA (nucleoside-2′-*O*-)-methyltransferase (hMTr2) (Werner et al., 2011) were identified.

The influenza type A virus RNA polymerase cleaves preferentially RNA with 5′ Cap 1 over that with Cap 0, but not RNA with an unmethylated core cap (Wakai et al., 2011). The RNA polymerase from the type B virus shows similar substrate specificity, with the exception that the type B RNA polymerase also cleaves RNA with an unmethylated core cap (Wakai et al., 2011). Consistent with this, the *in vitro* transcription activity of influenza virus is stimulated more by mRNA methylated at 2′-*O*-ribose residues than by mRNA lacking 2′-*O*-ribose methylation (Bouloy et al., 1980). Therefore, it is necessary to establish a procedure to prepare an RNA substrate with the Cap 1 structure for the development of a screening system for anti-cap-snatching agents.

To date, only a few studies have described an inhibitor of cap-snatching activity (Tomassini et al., 1994, 1996; Hsu et al., 2012). In these studies, the mRNA of alfalfa mosaic virus RNA 4 was first decapped *in vitro*, and then capped using enzymes purified from vaccinia virus to prepare the substrate. However, this complicated substrate preparation procedure and the heterogeneity of the natural RNA might hinder the efficient screening of candidate compounds.

During biochemical and molecular biological studies of the capping enzymes, we purified these enzymes from various sources, including mammalian cells, brine shrimp, and yeast, analyzed the biochemical mechanism of cap formation (reviewed in Mizumoto and Kaziro, 1987), and cloned the genes of these enzymes from humans (Tsukamoto et al., 1998a,b) and yeast (Shibagaki et al., 1992; Tsukamoto et al., 1997). Based on these studies, a novel method to produce a short Cap 1-RNA with 3′-biotinylation and a convenient streptavidin–biotin-based pull-down assay for screening anti-influenza-virus compounds that target the viral cap-snatching activity have been developed in this study. An RNA oligonucleotide with biotinylated uridine monophosphate (UMP) at its 3′ end was synthesized by *in vitro* transcription using T7 RNA polymerase, and was separated from any prematurely terminated products by C_{18} reverse-phase column chromatography. The Cap 1 structure was added to the full-length RNA with an efficiency of over 95% using three recombinant capping enzymes. This capped RNA was shown to be an efficient substrate for the influenza virus cap-snatching activity. A known inhibitor of the cap-snatching reaction (Tomassini et al., 1994) could be evaluated with this system, demonstrating the efficacy of the system for the screening of antiviral compounds.

2. Materials and methods

2.1. Influenza virus

Influenza virus A/PR/8/34 (H1N1) was kindly supplied by Dr. M. Yamasaki (Institute of Microbial Chemistry, Japan).

2.2. Recombinant enzymes

DNA fragments encoding the N-terminal domain of the vaccinia virus D1R subunit (D1N, amino acid residues 1–536, RNA 5′-triphosphatase and mRNA guanylyltransferase; GenBank accession: AGB75827.1), *S. cerevisiae* ABD1 ([guanine-*N*-]-methyltransferase; GenBank accession: AAA34383.1), and vaccinia virus VP39 ([nucleoside-2′-*O*-]-methyltransferase; GenBank accession: AAB19921.1) were PCR amplified from the full-length vaccinia virus D1R subunit DNA (a kind gift from Dr. S. Shuman [Memorial Sloan-Kettering Cancer Center, USA] (Shuman, 1990)), genomic DNA from *S. cerevisiae* NN1 (Yamagishi et al., 1995), and the genomic DNA of vaccinia virus WTF7-3 (kindly provided by Dr. T. Nakayama [Kitasato Institute for Life Sciences, Japan]), respectively, using following primers: D1N, D1Nf (5′-GGCATATGGATGCCAACGTAGTATC-3′) and D1Nr (5′-GGAAGCTTAATCCGATAGTTTATCCTC-3′); ABD1, yABDf (5′-CCAGTGCATATGTCAACCAAACCAG-3′) and yABDr (5′-CCCGGATCCTCAGTTGGGCTTTACGC-3′); VP39, VP39f (5′-GGCATATGGATGTTGTGTCGTTAGATAAACC-3′) and VP39r (5′-CCGGATCCTTATTTATTACCGCGTACGGATCTC-3′). The amplified fragments were digested with appropriate restriction enzymes (D1N, Nde I and Hind III; ABD1, Nde I and BamH I; VP39, Nde I and BamH I) and inserted into correspondingly digested pT7-7(His) (Shibagaki et al., 1997). The resultant plasmids, pHis(T)–D1N(1–536), pHis(T)–ABD1, and pHis(T)–VP39, respectively, were introduced into *Escherichia coli* BL21(DE3)pLysS (Studier et al., 1990). The transformed cells were grown in LB medium containing 50 μg/ml ampicillin at 25 °C. When OD_{600} reached 1.0, protein expression was induced with isopropylthiogalactoside (0.5 mM) and the cells were harvested after 5 h in culture at 25 °C.

The *E. coli* pellet (3 g) was suspended in 40 ml of lysis buffer (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride) and 0.5% Triton X-100. After lysis by sonication and clarification by centrifugation, the supernatant was applied to Ni^+-NTA agarose (Qiagen, Germany). The column was eluted with a gradient of imidazole (20–300 mM) in Buffer A (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 5 mM 2-mercaptoethanol, and 10% glycerol). D1N, ABD1, and VP39 were eluted at around 100 mM, 100–200 mM, and 100–150 mM imidazole, respectively. The fractions containing the recombinant enzymes were pooled, dialyzed against Buffer B (20 mM Tris–HCl [pH 7.0], 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol), and applied to a High-Trap SP Sepharose FPLC column (ø 0.4 cm × 2 cm; Amersham Biosciences, Sweden). The column was eluted with a linear gradient of NaCl (0.05–1.0 M) in Buffer B. D1N, ABD1, and VP39 were eluted at 200–300 mM, 300–400 mM, and 400–500 mM NaCl, respectively. The fractions containing the recombinant enzymes were pooled, dialyzed against Buffer C (20 mM HEPES-Na [pH 7.9], 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 50% glycerol), and stored in aliquots at −80 °C until use.

2.3. Synthesis of biotinylated Cap 1-RNA (m^7GpppGm-RNA)

A triphosphate-ended RNA (32 nucleotides: 5′-GAACAACAA-AAAAAACAACCAACAACCAAAU-biotin-3′), designated "$GACU_{32}$-biot", was synthesized using T7 RNA polymerase (Ambion, USA)