Cellular Human CLE/C14orf166 Protein Interacts with Influenza Virus Polymerase and Is Required for Viral Replication[▽]

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The influenza A virus polymerase associates with a number of cellular transcription-related factors, including RNA polymerase II. We previously described the interaction of influenza virus polymerase subunit PA with human CLE/C14orf166 protein (hCLE), a positive modulator of this cellular RNA polymerase. Here, we show that hCLE also interacts with the influenza virus polymerase complex and colocalizes with viral ribonucleoproteins. Silencing of hCLE causes reduction of viral polymerase activity, viral RNA transcription and replication, virus titer, and viral particle production. Altogether, these findings indicate that the cellular transcription factor hCLE is an important protein for influenza virus replication.

Influenza A virus contains eight single-stranded segments of negative-polarity RNA and encodes 11 proteins (9). Four of them are responsible for genome expression, the three polymerase subunits (PA, PB1, and PB2) and the nucleoprotein (NP). These proteins associate with each viral RNA segment to constitute the viral ribonucleoproteins (vRNPs) (9, 22). A functional coupling between viral and cellular transcription exists, due to the unusual viral initiation mechanism that uses as primers short-capped oligonucleotides scavenged from newly synthesized RNA polymerase II (RNAP II) transcripts. Within the viral polymerase, subunit PB1 contains the catalytic polymerase activity (4), cap recognition is achieved by subunit PB2 (5, 13, 27), and subunit PA is required to cleave the capped oligonucleotides (8, 29). In accordance with the viral transcription mechanism, a number of cellular transcription-related factors have been reported to associate with the viral polymerase complex and/or the polymerase subunits. Among these, the interaction with RNAP II itself should be emphasized (11). Other transcription-related factors found to interact with the viral polymerase are Ebp-1 (Erb-B3 binding protein 1) (14), which represses the transcription of cell cycle genes regulated by E2F transcription factors (21), DDX5 (16), a transcription coactivator that may play a role in cellular transcription initiation (3), and SFPQ/PSF factor (16), which stimulates cellular pre-mRNA processing (25). However, the individual biological mechanisms behind these host-cell interactions remain elusive in most cases.

hCLE interacts with the influenza virus polymerase complex. Using a yeast two-hybrid screening assay, we previously reported the interaction of human CLE (hCLE) and the chromatin remodeler factor CHD6 with the influenza virus polymerase subunit PA (15). Further characterization indicated that hCLE associates with and is a positive modulator of RNAP II (20). Hence, we wanted to determine whether hCLE interacted with the entire viral polymerase complex. HEK293T

cells were infected with the influenza virus A/WSN/33 (WSN) strain at a multiplicity of infection (MOI) of 3 PFU/cell, and at 6 h postinfection (h.p.i.), coimmunoprecipitation analyses were carried out. The infected cells were collected and lysed in a buffer composed of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.5% Igepal, and Complete protease inhibitor (Roche). The lysates were centrifuged at 10,000 × g, and the supernatants were used for immunoprecipitation studies with a rabbit polyclonal anti-hCLE antibody (15) or the pre-immune serum. The immunocomplexes were washed 10 times with a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 0.5 mM dithiothreitol, and 0.2 mM EDTA, and the coimmunoprecipitated proteins were analyzed by Western blotting as described previously (23). All three polymerase subunits associated with hCLE, whereas the pre-immune antibody did not immunoprecipitate any of them (Fig. 1A). Examination of *in vivo* colocalization of hCLE with components of the viral RNPs, such as NP and PB2, was also carried out. First, we examined whether changes in hCLE distribution take place during infection. Cultures of A549 cells were left uninfected or infected with the WSN strain at an MOI of 3 PFU/cell and at 6 h.p.i. were fixed and analyzed by immunofluorescence with antibodies against hCLE. As described previously (15), hCLE is distributed in the nucleus and the cytoplasm in either uninfected or infected A549 cells (Fig. 1B, left). Next, colocalization studies were performed using infected A549 cells that were stained with 4',6'-diamidino-2-phenylindole (DAPI) and incubated with a rabbit polyclonal anti-hCLE antibody (1:1,000) (15), a monoclonal anti-PB2 antibody (1:3) (2), and a rat polyclonal anti-NP antibody (1:2,000) (24). For these studies, single confocal sections and the colocalization mask that produces binary images showing only overlapping pixels (white spots) were used. In agreement with the observed association of hCLE with the viral polymerase, triple colocalization of hCLE with PB2 and NP was obtained (Fig. 1B, right, hCLE-PB2-NP). The data obtained indicate that hCLE interacts with the polymerase complex and colocalizes *in vivo* with viral RNPs.

Effect of hCLE silencing on influenza virus replication. The results presented above suggest that hCLE could be relevant

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FIG. 1. Interaction of hCLE with influenza virus polymerase. (A) Cultures of HEK293T cells were infected with influenza WSN virus and at 6 h.p.i., immunoprecipitation studies were carried out. The presence of hCLE, PB2, PA, and PB1 was monitored in Western blots. Input, HEK293T extracts; Mock, mock-infected cells; Flu, influenza virus-infected cells; α -Ctrl, immunoprecipitate with a control preimmune serum; α -hCLE, immunoprecipitate using a specific antibody against hCLE. (B) Left, cultures of mock-infected A549 cells or A549 cells infected with the WSN strain at 3 PFU/cell at 6 h.p.i. were processed for immunofluorescence assay using an antibody against hCLE (projections). Right, for confocal microscopy, single confocal sections of infected cells stained with DAPI to detect DNA and with antibodies against hCLE, PB2, and NP were used. The hCLE-PB2-NP panel shows the signals common to the corresponding three antibodies obtained with the colocalization mask. The bottom row shows magnified views of the cells marked with arrows.

for the influenza virus life cycle. To establish the role of hCLE protein during viral infection, RNA interference (RNAi)-mediated hCLE silencing experiments were performed. Cultures of HEK293T cells were transiently transfected by the calcium phosphate method (28) with a plasmid expressing a small hairpin RNA (shRNA) specific for hCLE (pSR-hCLE) (20) or a control plasmid expressing an irrelevant shRNA (pSR-TM) (6, 7). As shown in Fig. 2A, the expression of the hCLE-specific shRNA reduced hCLE protein accumulation by about 70% compared with the results with TM shRNA. Control or silenced cells were then used to carry out *in vivo* reconstitution of viral RNPs.

Since our previous studies indicated that hCLE silencing diminishes cellular mRNA transcription by up to 50% (20), we used an infection-transfection system in which reconstitution of recombinant RNPs was carried out in HEK293T cells that were first infected with a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) (12). The cells were then transfected with plasmids pGPB1, pGPB2, pGPA, pGNP, and pT7NSCAT-RT that encode the PA, PB1, PB2, and NP proteins from influenza A/Victoria/3/75 (VIC) strain and a negative-sense virus-like chloramphenicol acetyltransferase (CAT) RNA under the T7 promoter, respectively (18, 19). In control cells, the pGPA plasmid was omitted. CAT accumulation was used as a measure of the replication/transcription activity of

recombinant RNPs (Fig. 2B). The results show a 50% decrease in CAT accumulation in hCLE-silenced cells. Next, we examined the viruslike CAT positive- and negative-sense RNA levels produced by the reconstituted RNPs in control and transiently hCLE-silenced cells. Northern blot analysis using labeled *in vitro*-transcribed CAT sense and antisense RNAs as probes was performed (26). Important reductions in vRNA (45%) and positive-sense RNA (cRNA plus mRNA) (86%) were observed when the RNPs were reconstituted in hCLE-knockdown cells (Fig. 2C). As the amount of cRNA is much lower than that of mRNA, the data obtained would mainly represent mRNA levels. The numbers in Fig. 2B and C represent the means and standard deviations of three independent experiments. Therefore, silencing of hCLE inhibits viral RNA replication, as well as transcription.

To exclude a decrease in RNP accumulation that could account for the observed decrease in CAT activity and RNA transcription/replication, the expression of all RNP proteins and β -tubulin was determined in parallel by Western blotting. As can be seen by the results, similar RNP protein accumulation levels were obtained (Fig. 2D). The observed differences could be due to lower viral polymerase activity or to deficient RNP complex formation in hCLE-silenced cells. To distinguish between these possibilities, *in vivo* RNP reconstitution using a plasmid expressing a PB2-His-tagged subunit that does not

FIG. 2. Silencing of hCLE reduces influenza virus RNP activity. (A) HEK293T cells were transfected with pSR-hCLE or control pSR-TM plasmid, and silencing of hCLE was monitored in total cell extracts by Western blotting. As loading control, detection of β -tubulin (β -Tub) was carried out. (B) hCLE-silenced or control cells as described for panel A were used for *in vivo* RNP reconstitution. Forty-eight hours after reconstitution, the amount of CAT protein present in total cell extract was analyzed by enzyme-linked immunosorbent assay. Ctrl, plasmid expressing PA was omitted as a negative control; **, $P < 0.01$ (t test). (C) Aliquots of the samples used in the experiment whose results are shown in panel B were analyzed for negative and positive CAT RNAs using Northern blots with labeled *in vitro*-transcribed sense and antisense CAT RNAs as probes. (D) Aliquots of the samples used in the experiment whose results are shown in panel B were also analyzed for the presence of RNP proteins and β -tubulin by Western blotting. (E) hCLE-silenced or control cells as described for panel A were used for *in vivo* reconstitution of RNPs containing a His-tagged PB2 subunit. Forty-eight hours after reconstitution, the RNPs were purified by chromatography on Ni-NTA-agarose, and the presence of RNP proteins in the eluted fractions was analyzed by Western blotting. Ctrl, plasmid expressing PB2-His was omitted as a negative control; Input, around 1% of the total HEK293T extracts; E1 and E2, eluted fraction 1 and 2, respectively, each representing around 8% of the eluted proteins.

alter the replication activity of the viral polymerase but allows its affinity purification (1) was performed. Viral RNPs were then reconstituted in hCLE-silenced or control cells and purified by affinity chromatography on a Ni^{2+} -nitrilotriacetic acid (NTA) agarose resin and analyzed by Western blotting (Fig. 2E). As expected, the omission of the PB2-His-expressing plasmid prevented RNP purification (Ctrl). When all plasmids were included in the reconstitution assay, RNPs were purified, and the amounts of RNP complexes were similar both in control and hCLE-silenced cells (RNPs). Altogether, these data indicate that hCLE is not essential for RNP complex formation but positively modulates viral polymerase activity.

To analyze the relevance of hCLE in virus infection, we constructed A549 and HEK293T stable cell lines by transfection with the pSR-puro-TM or pSR-puro-hCLE plasmids using Lipofectamine 2000 (Invitrogen), followed by selection with 0.5 $\mu\text{g}/\text{ml}$ or 2 $\mu\text{g}/\text{ml}$ of puromycin, respectively. Both A549

and HEK293T silenced cell populations presented an overall 60% decrease in hCLE accumulation compared with the hCLE accumulation in control cells transfected with the TM plasmid (Fig. 3A). Under these conditions, we evaluated the effect of hCLE silencing on virus replication by infecting the cells at a low multiplicity of infection (10^{-3} PFU/cell). A decrease in viral protein accumulation (Fig. 3B) and a reduction of around 1 log in viral titers were obtained in both types of hCLE-deficient cells compared with the results for TM plasmid-transfected cells (Fig. 3C), indicating that cellular hCLE protein is necessary for influenza virus replication.

The reduced viral titer observed after multiple rounds of viral replication could be due to lower virion production during the successive replication events, lower infectivity of the viral particles, or both. To examine these possibilities, we quantified the amounts of viral proteins present in the supernatants of the stably silenced or control cell lines infected at a low MOI (10^{-3}

FIG. 3. hCLE silencing reduces the viral titer in multiple-step growth experiments. (A) HEK293T and A549 stably hCLE-silenced (hCLE) or control (TM) cells were used, and silencing of hCLE and level of β -tubulin as loading control were monitored in total cell extracts by Western blotting. (B) Cultures of stably hCLE-silenced or control silenced cells were infected at 10^{-3} PFU/cell with the WSN strain of influenza virus. At 72 (HEK293T) or 60 (A549) h.p.i., cell extracts were taken and PB1, PA, NP, M1, M2, and β -tubulin proteins were analyzed by Western blotting. Quantitation of proteins present in hCLE-silenced cells compared with their levels in control cells is indicated. (C) Aliquots of the culture supernatants of the infected cells at 10^{-3} PFU/cell were collected at the indicated times postinfection, and the virus titers were determined by plaque assay in MDCK cells. Left, HEK293T cells; right, A549 cells.

PFU/cell). At 60 (HEK293T) or 48 (A549) h.p.i., supernatants were collected. Equal amounts of culture supernatants were precleared by centrifugation at 10,000 rpm for 10 min at 4°C, and the supernatants were centrifuged on a 33% sucrose cushion at 26,000 rpm for 2.5 h. The pellets were resuspended in TNE buffer (100 mM NaCl, 5 mM EDTA, and 50 mM HCl-Tris, pH 7.5) and centrifuged over a 33%-to-50% sucrose step gradient for 60 min at 40,000 rpm and 4°C. The 33%-to-50% interface was collected, diluted in the same buffer, and further centrifuged through a 33% sucrose cushion for 60 min at 40,000 rpm and 4°C. The purified virion composition was analyzed by Western blotting, and the results are shown in Fig. 4. Reduced amounts of viral proteins (NP, M1, and M2) were detected in the supernatants of silenced cells compared with the amounts in the corresponding control cells. Quantitative analysis showed that these reductions were around 50 to 70%, whereas a 10-fold decrease in viral titer was observed in hCLE-silenced cells (Fig. 3), suggesting a loss of infectivity of the virions generated in this situation. hCLE has an important role in modulating cellular transcription (20), but an additional function in mRNA metabolism has also been suggested (10, 17). The protein has a nuclear and cytoplasmic intracellular localization (15) and is a component of neuronal mRNA-trans-

porting granules that allow the localized translation of specific mRNAs (10, 17). Thus, the possible reduction in virion infectivity observed in hCLE-silenced cells could be the result of a defective early viral event, such as transport of incoming

FIG. 4. Production of viral particles is reduced in hCLE-silenced cells. Cultures of stably hCLE- or control (TM)-silenced HEK293T (A) or A549 (B) cells were infected at 10^{-3} PFU/cell with the WSN strain of influenza virus. At 60 (HEK293T) or 48 (A549) h.p.i., supernatants were collected and equal amounts of HEK293T and A549 culture supernatants were used for virion purification. The purified virion composition was analyzed by Western blotting with anti-NP, -M1, and -M2 antibodies. Quantitation of proteins present in hCLE-silenced cells compared with their levels in control cells is indicated (right).

vRNPs to the nucleus or early viral transcription. Additional studies will provide clues about the molecular mechanisms implicated in the process.

Here, we demonstrate that hCLE protein, which interacts with both RNAP II and viral RNA polymerase (Fig. 1) and also positively modulates their activities (reference 20 and Fig. 2, respectively), is an important cellular factor for influenza virus replication. It is worthwhile to remember that viral transcription must be functionally associated with cellular transcription, as 5'-capped oligonucleotides are required as primers for viral transcription. In agreement with that, it has been shown that influenza virus polymerase binds to RNAP II engaged in transcription initiation (11). The presence of hCLE in RNAP II-containing complexes could have a cooperative role in that functional association between viral and cellular transcription machineries. Since we also show that hCLE is required for viral RNA replication (Fig. 2C), it is possible that a coupling between viral and cellular transcription machinery may modulate RNA replication, besides affecting viral transcription. In addition, hCLE could work as an RNA replication factor independently of its role as a cellular transcription modulator. Finally, the described properties of hCLE as a positive modulator of influenza virus suggest that this cellular factor could be a potential candidate to design new antiviral compounds by targeting the hCLE-PA interacting domains.

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