Transfection of COS Cells with Human Cystatin cDNA and Its Effect on HSV-1 Replication[a]

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INTRODUCTION

The cystatin (Csn) superfamily of proteins are natural cysteine proteinase (CysP) inhibitors; however, their rate of inhibition of known CysP varies greatly among the different members. These variations may allow for a more vast defense mechanism against diseases of bacterial and/or viral origin. Several Csns have been shown to possess viral inhibitory properties. These include chicken egg white (CEW) Csn and oryzacystatin (rice Csn) and the ability to inhibit poliovirus replication.[1,2] Oryzacystatin has also been shown to inhibit herpes simplex virus Type 1 (HSV-1) *in vitro* and *in vivo*.[3] Human cystatin C (CsnC) has the ability to inhibit HSV-1 and coronavirus replication.[4,5] Human whole saliva and salivary fractions containing CsnSN have been shown to inhibit HSV-1 replication.[6,7] Thus we are interested in determining the mechanism of inhibition of HSV-1 by CsnC and CsnSN. We have shown that rCsnSN, and confirmed that rCsnC, reduce the viral yield of HSV-1 when these proteins are added exogenously to CV-1 cells at the start of HSV-1 infection.[8] It is thought that the Csns may enter the cells after the cell membranes become permeable as a result of the viral infection[4] and are thus able to inhibit viral and/or host cell CysP necessary for the viral replication. Poliovirus replication is inhibited by CEW Csn through a CysP necessary for polyprotein processing and essential for viral replication and propagation.[1] Also, a serine proteinase encoded by HSV-1 has been identified and found to be necessary for the packaging of the mature virions.[9] Therefore, it is plausible that CsnC and/or CsnSN inhibit HSV-1 replication through a CysP.

We hypothesize that if Csn is produced in large amounts by the cells prior to or during the viral infection, then it may more effectively inhibit HSV-1 replication. Our hypothesis is supported by a recent study that showed that the introduction of the corn Csn gene into rice plants resulted in high levels of expression of the corn Csn mRNA and protein. The corn Csn produced in rice served as an effective inhibitor of insect pest gut CysP.[10] To evaluate our hypothesis, we are using transfection technology for establishing CV-1 cell lines that will continuously produce CsnSN and CsnC mRNA and protein, with a goal of using these cell lines for HSV-1 inhibition studies. These studies will allow us to better understand the mechanism of interaction and thereby inhibition of HSV-1 by Csns.

RESULTS AND DISCUSSION

In order to establish cell lines expressing high levels of Csn for HSV-1 inhibition studies, we have initially cloned cDNA fragments encoding the secreted peptide of

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CsnC and CsnSN (120 and 121 amino acids, respectively) into the eukaryotic expression vector pcDNA 3.0, under the control of the CMV promoter. The recombinant plasmids were transfected into COS-1 cells. Stable clones were selected with G418 and isolated onto 24-well plates. Simultaneous to individual clone selection, we analyzed a heterogeneous population of cells for mRNA and protein production. The Northern blot analysis showed a very large increase in mRNA of both CsnC and CsnSN (refer to FIG. 1A), whereas the Western blot analysis was inconclusive (data not shown). Upon evaluation of individual clones (>20 for each of CsnC and SN), we found no increase in the Csn mRNA or protein production in any of the clones. These results indicate that even though the individual clones were antibiotic resistant, they were false positive.

In order to better evaluate the ability to transfect eukaryotic cells with CsnC or CsnSN, we used transient transfections. We examined both lipofectamine and calcium phosphate transfection methods, as well as CV-1 and NIH-293 cells. We found that both CV-1 and NIH-293 transfected best with lipofectamine and that the NIH-293 cells have little or no endogenous CsnC (refer to FIG. 1B). As compared to CV-1 cells, NIH-293 cells were less infectable by HSV-1; therefore, in subsequent transfections and HSV-1 infections we used CV-1 cells. We have successfully transiently transfected CV-1 cells with untargeted CsnC cDNA, as documented by an increase in CsnC protein production by Western blot analysis of total cell protein (as shown in FIG. 2A). However, transiently transfected CV-1 cells with untargeted CsnSN cDNA have shown no increase in CsnSN protein production (data not shown); the reason for this remains un-

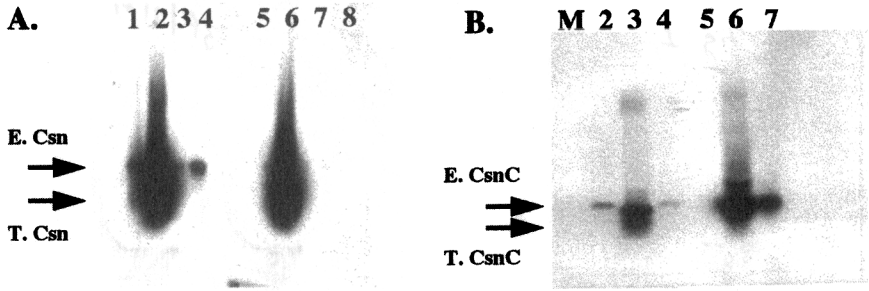

FIGURE 1. Northern blot analyses of CsnC and CsnSN mRNAs in transfected cells. **A:** Analysis of Csn mRNAs in a heterogeneous population of stably transfected COS-1 cells. Lanes 1 and 5: cells transfected with pcDNA 3.0 vector only; lanes 2 and 6: transfected cells with CsnC and CsnSN, respectively; lane 3: human ovary RNA (CsnC positive control); lane 7: human submandibular gland RNA (CsnSN positive control); lanes 4 and 8: untransfected COS-1 cells. Lanes 1–4 were probed with a CsnC probe, and lanes 5–8 with a CsnSN probe. E. Csn represents endogenous Csn, 360 b; T. Csn represents transfected Csn, ~750 b. **B:** Analysis of CsnC mRNA in transiently transfected CV-1 cells (lanes 2–4) and NIH-293 cells (lanes 5–7). Lane 1: RNA marker; lanes 2 and 5: untransfected cells; lanes 3 and 6: cells transfected with CsnC using the lipofectamine method; lanes 4 and 7: cells transfected with CsnC using calcium phosphate method. Cells were harvested 48 h after transfections. Methods: The cells were transfected using either the lipofectamine-mediated transfer (20 mg) or the calcium phosphate method with CsnSN or CsnC cDNA (10 mg). Stable transfectants were selected with 800 mg/mL G418 (Geneticin). One plate containing transfected cells was used for extraction of total RNA. The RNA concentration was determined spectrophotometrically and confirmed by gel electrophoresis. The RNA (10 μg) was separated on a 1.0% agarose gel containing 2.0% formaldehyde, and a capillary transferred overnight onto a BA85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The blots were probed with a ^{32}P-labeled CsnC or CsnSN cDNA probe (360 bp fragment encoding the translated region).

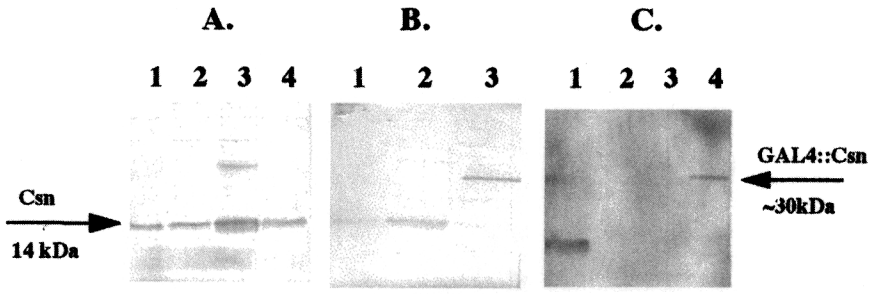

FIGURE 2. Western blot analyses of proteins from transiently transfected CV-1 cells. **A:** Analysis of cells transfected with untargeted CsnC. Lane 1: rCsnC (positive control); lane 2: untransfected cells; lane 3: cells transfected with CsnC using the lipofectamine method; lane 4: cells transfected with CsnC using the calcium phosphate method. **B:** Analysis of cells transfected with CsnC + GAL4 nuclear signal. Transfections were done using the lipofectamine method. Lane 1: rCsnC (positive control); lane 2: untransfected cells; lane 3: cells transfected with CsnC + GAL4 nuclear signal. **C:** Analysis of cells transfected with CsnSN +/– GAL4 nuclear signal. Transfections were done using the lipofectamine method. Lane 1: CsnSN (positive control); lane 2: untransfected cells; lane 3: cells transfected with untargeted CsnSN; lane 4: cells transfected with CsnSN + GAL4 nuclear signal. Methods: CV-1 cells were transfected with the lipofectamine or the calcium phosphate method, followed by incubation for 48 h. The total protein was then extracted in a 1× SDS-PAGE sample buffer containing 5.0% β-mercaptoethanol. Protein concentration was determined by a BIORAD protein assay. Proteins (50 μg) were separated on a 0.1% SDS-13% PAGE and transferred onto an Immobilon-P membrane (Millipore Corp. Bedford, MA) using semidry apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membranes in panels A and B were probed with a rabbit anti-CsnC (Dako Corp, Carpinteria, CA, 1:1000) and with rabbit anti-CsnSN (#221, 1:500, gift from Dr. Michael Levine, SUNY Buffalo) in panel C. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega Corp., Madison, WI, 1:7500) was used as a secondary antibody.

clear. Further, we have successfully transiently transfected CV-1 cells with CsnC and CsnSN cDNA containing a nuclear retention signal (147 aa residues of GAL4-encoding sequence[11] added to the 5′ end of the Csns), as reflected by a production of GAL4/CsnSN and GAL4/CsnC proteins, shown in FIGURE 2B and C, respectively. We targeted the nucleus because most HSV-1 functions (*i.e.*, transcription, replication, and packaging) occur within the host cell nucleus.

Currently, we are in the process of reselecting stable clones expressing high levels of rCsnC and rCsnSN ± nuclear signal. However, for these experiments we have subcloned CsnC and CsnSN cDNA fragments into the pcDNA 3.1 eukaryotic expression vector because the stable clones are selected with zeocin (250 μg/mL), which should yield less false positives and shorten the selection time.

We have also explored the possibility of HSV-1 inhibition using the transiently transfected CV-1 cells with CsnSN. The cells were infected with HSV-1 (m.o.i. of 0.1) at 24, 36, and 48 h posttransfection (p.t.). The results indicate that at 48 h p.t. we have achieved a similar degree of HSV-1 inhibition as compared with the exogenously added rCsnSN (~ 1 log of HSV-1 virus yield reduction, data not shown). This is probably because in transient transfections, only a small fraction of the cells become transfected, and the level of transfection is not consistent between each transfection. Therefore, it is necessary to obtain stable cell lines that express high amounts of CsnC or CsnSN in order to evaluate the mechanism of HSV-1 inhibition by Csns.

In conclusion, our first attempt to establish stable cell lines producing CsnSN or CsnC were unsuccessful. This may be due to a large incidence of false positives, or perhaps because it is not possible to produce stable cell lines expressing high levels of Csns (CysP inhibitors). In fact, it has been shown that it is very difficult to obtain transfected cells with expression levels of calpain (CysP) and/or its natural inhibitor, calpastatin, significantly different from that of control cells. Large deviations from the normal level seem to be lethal.[12] It is also documented that rice plants transfected with the rice Csn gene did not express high levels of rice Csn mRNA or protein (insufficient for the effective protection against insect pests).[13] On the other hand, the study by Irie *et al.*[10] has shown that transfection of the rice plants with the corn Csn gene yielded transgenic rice plants with levels of corn Csn protein more than 20 times higher than that of the intrinsic oryzacystatin. More importantly the corn Csn protein effectively inhibited insect pest gut CysP. Therefore, we remain optimistic that we will be successful in establishing cell lines that will produce high levels of CsnSN and CsnC that can be used to determine the mechanism of HSV-1 inhibition by cystatins.

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