Brief Report

Establishment of a new cell line for performing sensitive screening of nuclear export inhibitors

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ABSTRACT: Cell nuclear import and export of proteins are among the most important processes for cellular homeostasis. Viruses, including influenza virus and HIV-1 also utilize nuclear export machinery for their propagation. Therefore, the specific inhibition of nuclear export can be a good target for the development of antiviral drugs. This report documents the establishment of Madin-Darby Canine Kidney (MDCK) cells stably expressing GFP-NES fusion protein for the sensitive screening of novel nuclear export inhibitors. Leptomycin B (LMB), an inhibitor for the interaction between substrate nuclear export signal (NES) and cellular export factor, CRM1, was used for evaluating the screening system. The low concentration (30 pg/mL) and short time treatment (~10 min) of LMB resulted in the nuclear accumulation of GFP fluorescence.

***Keywords**:* Nuclear export, Screening, Antiviral drug

Introduction

The transport of proteins through nuclear pore complexes (NPCs) is mediated by importins and exportins (*1*). CRM1/exportin1 is the most characterized exportin. The formation of exportin-substrate molecule complexes is regulated by Ran. The export substrates of CRM1 possess a leucine-rich nuclear export signal (NES). The NES was identified in Heat shock cognate protein 70 (Hsc70), influenza virus NS2, HIV-1 Rev (*2*), protein kinase A inhibitor (*3*) (Figure 1) and other proteins.

A virus is a unique pathogen which is incapable of replicating without a host cell. It utilizes the host cell environment and cellular factors for its propagation (for a review, see *Ref. 4*). Nuclear export of proteins from the nucleus seems to be an attractive target for antiviral therapy because viruses such as human immunodeficiency virus (HIV) and influenza virus utilize this environment. Development of antiviral drugs is one of the most important strategies, however, some viruses, especially RNA viruses, easily resist drugs designed for virus gene products. It is well recognized that influenza virus, which is resistant to amantagine (the inhibition of viral M2 protein), are already spreading all over the world. The same problems are serious in HIV treatment, and a recent protocol, termed HAART therapy, a multi drug combination theory, is essential to treat patients with HIV. To make such antiviral therapy more effective, further development of novel drugs which target cellular proteins is essential. Therefore, inhibitors which control or inhibit the nuclear export system could be good candidates. However, only leptomycin A (LMA) and leptomycin B (LMB) are available for research use as inhibitors of CRM1-mediated nuclear export.

Recently, Hsc70 was identified to be a mediator for nuclear export of influenza viral ribonucleoprotein complex (RNP). Hsc70 has been found to possess NES (*5*) and involve nuclear export of importin and transportin (*6*). These findings also suggest that the inhibition of nuclear export of influenza viral RNP by LMB is effective for reducing influenza virus production (*7*). Although LMB is effective *in vitro*, it can also damage the cellular activity.

Recently a new cell line expressing GFP fused HIV-1 protease was established for screening anti-HIV protease activity (*8*). This report describes the establishment of a stable cell line which expresses the GFP - nuclear export signal (NES) fusion protein. The established cells (GES5) were sensitive to LMB. This cell line can be utilized to develop a new, high-throughput screening system for nuclear export inhibitors without using free virus.

Materials and Methods

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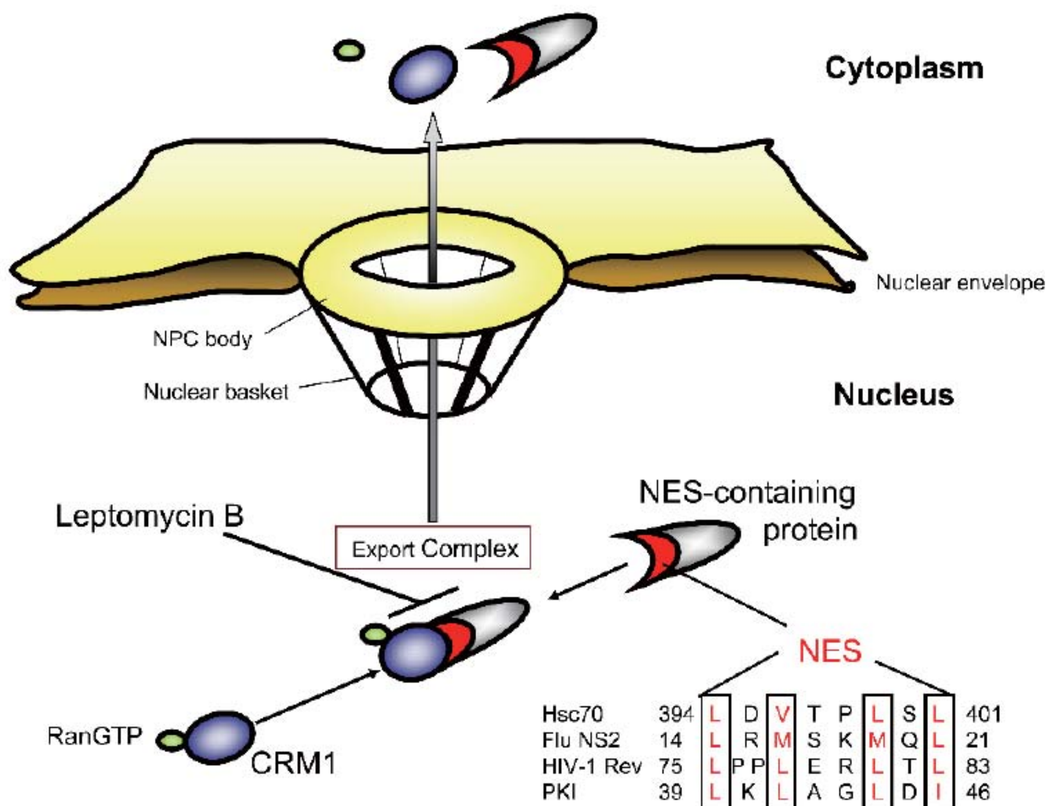

Figure 1. The general mechanism of nuclear export and representative NES-containing proteins. NES-containing protein binds to CRM1. Leptomycin B inhibits NES-CRM1 interaction. CRM1 is an Importin-beta family protein and binds to Ran-GTP. The complex is exported from the nucleus to cytoplasm through NPC. The dissociation of NES containing protein-CRM1-Ran-GTP complex is triggered by conversion of Ran-GTP to Ran-GDP. The representative leucine-rich NES are also shown.

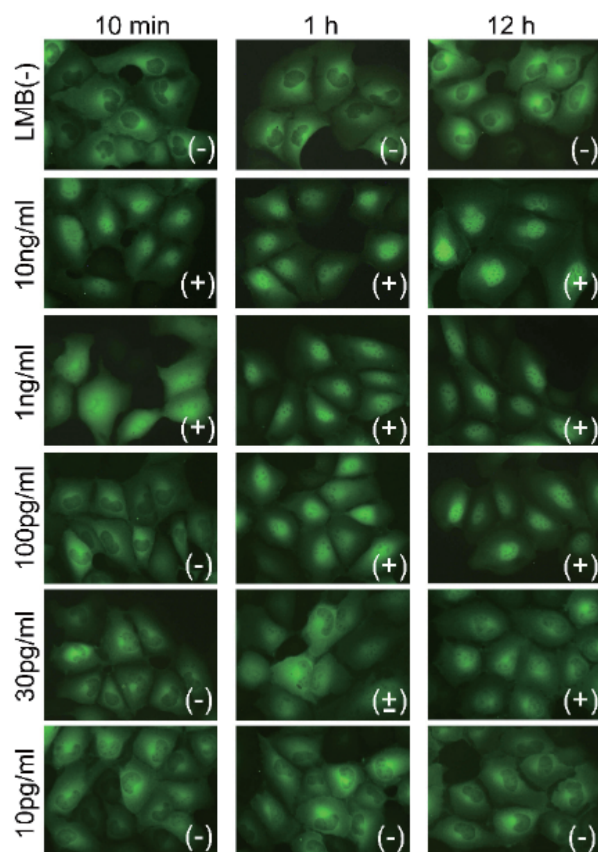

Figure 2. LMB sensitivity of GES5 cells. GES5 cells were treated with LMB as indicated. GFP fluorescence accumulates at 10 min after addition of 10 ng/mL or 1 ng/mL of LMB and is retained in the nucleus up to 12 h, whereas very low concentration of LMB treatment (100 pg/mL or 30 pg/mL) requires 1 h for nuclear accumulation of GFP fluorescence. The nuclear accumulation of GFP fluorescence is shown as (–), (±) and (+).

Cells, chemicals and plasmids

Madin-Darby canine kidney (MDCK) cells were maintained in MEM supplemented with 5% FBS. LMB was purchased from LC Laboratories, Inc. (Woburn, MA, USA) and dissolved in 70% ethanol to a final concentration of 10 μg/mL and stored at -20°C. A plasmid for expressing GFP-NES fusion protein (pEGFP-NES) was kindly supplied by Dr. Shoko Saitoh (Tsukuba University, Japan) (*9*). For the establishment of cell lines, ~90% confluent MDCK cells were transfected in a 24 well plate with 2 μg of pEGFP-NES in the presence of 6 μL of lipofectamine 2000 (Invtrogen). The cells were incubated for 5 h with the DNA-lipofectamine 2000 complex, and then were washed once with MEM, and replaced with MEM containing 5% FBS. Twenty-one hours later, the cells were trypsinized and seeded into 90 mm dishes. G418 sulfate (Nacalai, Kyoto Japan) was added to a final concentration of 600 μg/mL. The cell lines expressing high intensity fluorescence were selected under fluorescence microscopy (Axiovert25, Carl Zeiss).

Nuclear translocation assays using Leptomycin B (LMB)

GES5 cells were grown on coverslips, and LMB was added at the final concentrations indicated in Figure 2. The accumulation of fluorescence into the nucleus was observed under fluorescence microscopy (Axiophot, Carl Zeiss).

Results and Discussion

Establishment of a cell line which expresses GFP-NES fusion protein

To establish cell lines stably expressing GFP-NES fusion protein, MDCK cells were transfected with pEGFP-NES and colonies were selected in the presence of G418 sulfate. A cell line, designated MDCK-GFP-NES#5 (GES5) was selected, and tested for LMB sensitivity (Figure 2). Fluorescence of GFP was diffusely distributed in both the nucleus and cytoplasm (data not shown), whereas that of GFP-NES clearly distributed in cytoplasm (Figure 2). When LMB was added, CRM1-dependent nuclear export was inhibited, thus resulting in nuclear accumulation of GFP-NES protein. This effect was observed at 10 min after addition of LMB.

This cell line system is sensitive to LMB and it is safe, because there is no need to use live HIV-1 or influenza virus. The newly established cell line, GES5, was easily maintained with conventional medium and serum. The addition of nuclear export inhibitors such as LMB rapidly results in nuclear accumulation of GFP fluorescence. Indeed, when the MDCK cell line expressing GFP-Hsc70 and GFP-Hsc54 (*5*) were tested for LMB sensitivity, accumulation of fluorescence in nucleus requires 24 h and 6 h, respectively (data not shown). Moreover, LMB is generally used at a concentration of 5-10 ng/mL as reported previously (*5,9,10*). In this system, nuclear accumulation of GFP fluorescence is observed with a 30 pg/mL treatment (Figure 2). Therefore, the GES5 cell line is thus considered to be useful for a sensitive and high-throughput screening system.

Acknowledgements

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