Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

ACADEMIC PRESS

Available online at www.sciencedirect.com
SCIENCE DIRECT®

VIROLOGY

Virology 306 (2003) 225–235

www.elsevier.com/locate/yviro

Identification of nucleolus localization signal of betanodavirus GGNNV protein α

Yan Xiang Guo, Klara Dallmann,[1] and Jimmy Kwang*

Temasek Life Sciences Laboratory, The National University of Singapore, Singapore 117604

Received 12 June 2002; returned to author for revision 8 July 2002; accepted 13 August 2002

Abstract

Betanodavirus greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV) protein α, a virus capsid protein, was detected in both nucleolus and cytoplasm of infected cells of Asian sea bass (SB) and transfected cells of SB and Cos-7 with pcDNA3.1/RNA2. To study its subcellular localization, ORF of protein α with 338 aa was fused with enhanced green fluorescent protein (EGFP) gene and was detected in transfected cells in the absence of other viral proteins. In both SB and Cos-7 cells, protein α was found to localize EGFP to the nucleolus and cytoplasm. Deletion mutants of protein α indicated that N-terminal 43 amino acid residues were required to import EGFP-α protein into the nucleolus. Further deletions within the 43 amino acid backbone, EGFP/33aa(1–33) and EGFP/30aa(14–43), localized to the nucleolus, suggesting that the 20 amino acids from 14 to 33 of protein α were the domain of nucleolus localization. To further determine the nucleolus targeting sequence, deletion mutations within the 20 amino acids of protein α were constructed. It was found that the deletion of ^{23}RRR^{25}, ^{29}RRR^{31}, or ^{23}RRRANNRRR^{31} prevented the accumulation of EGFP fusion proteins into the nucleolus, demonstrating that ^{23}RRRANNRRR^{31} contain the signal required for nucleolar localization. A similar distribution pattern of localization of protein α and its deletion mutants in SB and Cos-7 cells suggested that N-terminal residues of protein α ^{23}RRRANNRRR^{31} constitute a nucleolus localization signal that functions in both fish and mammalian cells.
© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Betanodavirus greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV); Capsid protein α; Enhanced green fluorescent protein (EGFP); Nucleolus localization; Deletion mutations; Nucleolus localization signal; SB and Cos-7 cells

Introduction

Nodaviruses are small, nonenveloped, spherical viruses with bipartite positive-sense RNA genomes, which are capped but not polyadenylated. Two genera have been distinguished in the *Nodaviridae* family: alphanodaviruses, which are originally isolated from insects and betanodaviruses, which usually infect a wide variety of larval and juvenile marine fish (Ball et al., 1994, 2000). Similar to alphanodaviruses, the genome of betanodaviruses has two coencapsidated genomic RNA segments (RNAs 1 and 2), both of which are required for infectivity. RNA1 encodes protein A, which is the viral component of the viral RNA-dependent RNA polymerase (RdRp). RNA2 encodes protein α, a proteolytic precursor of viral capsid proteins. A subgenomic RNA3 is synthesized during RNA replication from 3′ terminus of RNA1 and encodes one or two small proteins (B1 and B2) with unknown functions. As a member of betanodaviruses, the complete sequence of greasy grouper (*Epinephelus tauvina*) nervous necrosis viruses (GGNNV) of Singapore strain has been determined in our laboratory. The complete nucleotide sequence of RNA1 is 3103 nucleotides containing an ORF of 982 aa and the RNA2 is 1433 nucleotides containing an ORF of 338 aa (Tan et al., 2001). RNA2 encodes a 37-kDa capsid protein whose recombinant protein α could be used as a sensitive reagent to diagnosis GGNNV infection (Huang et al., 2001). In our previous work, GGNNV was demonstrated to be

* Corresponding author. Temasek Life Sciences Laboratory, 1 Research Link, The National University of Singapore, Singapore 117604. Fax: +65-6872-7007.

E-mail address: kwang@tll.org.sg (J. Kwang).

[1] Present address: Agricultural Biotechnology Center at Godollo, H-2101, P.O. Box 411, Godollo, Hungary.

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(02)00081-8

capable of inducing caspase-dependent apoptosis in GGNNV-infected SB cells and protein α was a possible apoptotic inducer in transfected cells (data not shown).

Nucleolus is mainly the site of ribosome biogenesis, a highly complex process leading to the production of preribosomal particles, which are then released to the nucleoplasm and exported to the cytoplasm as mature ribosomal subunits (Carmo-Fonseca et al., 2000). However, more and more exogenous nonribosomal proteins were demonstrated to be transported to the nucleolus under certain conditions. Proteins from porcine reproductive and respiratory syndrome virus (Rowland et al., 1999), Semliki Forest virus (Favre et al., 1994), coronavirus (Hiscox et al., 2001), and Borna disease virus (Pyper et al., 1998) have been reported to localize in the nucleolus and to get involved with various aspects of virus replication. It has been found that a cluster of basic amino acids could lead proteins to the nuclear or nucleolus (Kaffman and O'Shea, 1999).

In the past few years, many studies have shown that the green fluorescent protein (GFP) from jellyfish is a popular tool for localization of a specific protein distribution inside cells when the GFP is fused with that particular protein (Misteli and Spector, 1997; White and Stelzer, 1999; Hiscox et al., 2001; Shih and Lo, 2001). In this study, the protein α of GGNNV could be detected in both nucleolus and cytoplasm of SB cells infected by GGNNV and SB and Cos-7 cells transfected with pcDNA3.1/RNA2. The ORF of protein α was then fused with EGFP and transfected to the SB and Cos-7 cells to study its subcellular localization. It was found that the GGNNV protein α could direct the EGFP to the nucleolus of both SB and Cos-7 cells. Employing a series of deletion mutations, the N-terminal amino acid residues ^{23}RRRANNRRR^{31} were identified as a nucleolus localization signal which functioned in both fish and mammalian cells, indicating that there is a conserved mechanism for nucleolus localization in both cells.

Results

Localization of protein α in Asian sea bass (SB) cells infected with GGNNV and in SB and Cos-7 cells transfected with pcDNA3.1/RNA2

To identify the localization of protein α in SB cells infected with GGNNV, SB cells were fixed at 20 h postinfection and analyzed by immunofluorescence assay (IFA). A polyclonal guinea pig antiprotein α sera was used as primary antibody, followed by FITC-conjugated secondary antibody, and the cells were observed under fluorescence microscope. In the case of SB cells, DAPI was used to stain the nuclear boundary to visualize the nucleolus localization of protein α. IFA staining observations of SB cells exhibited two patterns on the localization of protein α, one with nucleolus and cytoplasmic staining (Fig. 1, images a–c) and the other without nucleolus labeling (data not shown). About 10% of GGNNV-infected SB cells showed protein α localization in both the nucleolus and the cytoplasm.

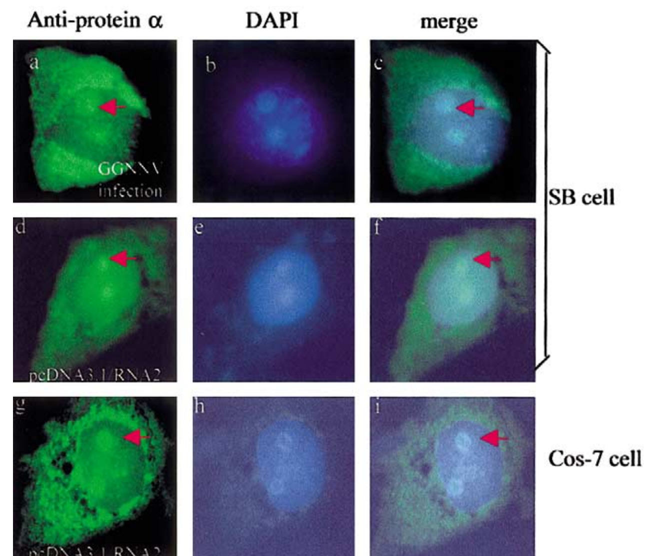

Fig. 1. Subcellular localization of protein α in cells infected with GGNNV and transfected with pcDNA3.1/RNA2. SB cells infected with GGNNV (images a–c) were fixed at 20 h postinfection and IFA was carried out to detect the expression and localization of protein α with the antibody against protein α. SB (images d–f) and Cos-7 (images g–i) cells transfected with pcDNA3.1/RNA2 were fixed at 36 h posttransfection and IFA was carried out to detect the expression and subcellular localization of protein α. DAPI staining was used to show nucleus. The merged image represents a superimposition of green and blue signals. The red arrows indicate nucleoli. The images were viewed with a Leica 550 IW fluorescence microscope and photographed in $\times$1000 amplification.

To investigate further the subcellular localization of GGNNV protein α in the absence of other viral proteins, the ORF cDNA of protein α was cloned into the eukaryotic expression vector pcDNA3.1(+) to construct pcDNA3.1/RNA2. SB cells transfected with pcDNA3.1/RNA2 were fixed at 36 h posttransfection and IFA was carried out with polyclonal guinea pig antiprotein α sera. Similarly, Cos-7 cells were transfected with pcDNA3.1/RNA2 to detect the localization of protein α in mammalian cells. In both SB (Fig. 1, images d–f) and Cos-7 cells (Fig. 1, images g–i), protein α was observed in both nucleolus and cytoplasm. The percentage of transfected cells exhibited in both the nucleolus and the cytoplasmic protein α localization is about equal ($\sim$10%) to that of the GGNNV-infected SB cells.

Localization of enhanced green fluorescent protein (EGFP)-α fusion protein in transfected cells

To investigate whether the GGNNV protein α could transport an exogen protein to the nucleolus, ORF cDNA of protein α was inserted into pEGFP-C1 to construct pEGFP/

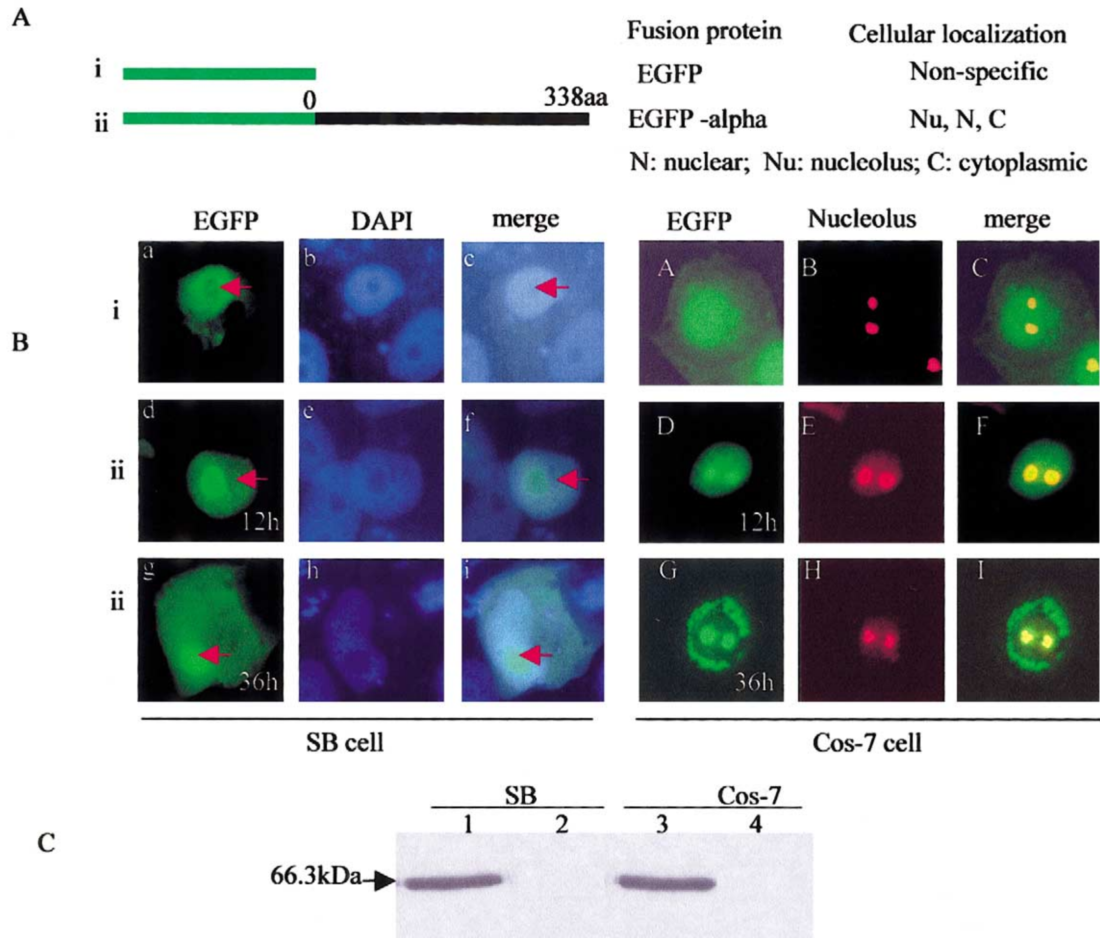

Fig. 2. (A) EGFP, EGFP-α fusion protein, and their cellular localization. Green bar indicates EGFP and black bar indicates protein α. (B) Localization of EGFP-α fusion protein in SB and Cos-7 cells. SB or Cos-7 cells were transfected with pEGFP-RNA2 plasmid with lipofectamine plus reagents on Chamber slides. SB cell expressing EGFP-α fusion protein (images d and g, green), nuclear image stained with DAPI (images e and h, blue), and merged signals are shown (images f and i) at 12 and 36 h posttransfection. The merged image represents a superimposition of green and blue signals. The images were viewed with a Leica 550 IW fluorescence microscope and photographed in ×1000 amplification. Cos-7 cell expressing EGFP-α fusion proteins (images D and G, green), nucleolus labeled with an antinucleolin mAb (images E and H, red), and merged signals (images F and I) are shown at 12 and 36 h posttransfection. The merged image represents a digital superimposition of green and red signals, where areas of fluorescence colocalization are yellow-orange. Cos-7 cells were viewed under a confocal microscope. Amplification, ×630. In addition, the distribution of EGFP in SB and Cos-7 cells was shown in image a and A, respectively. (C) Expression analysis of protein α by Western blot. SB (lane 1 and 2) or Cos-7 (lane 3 and 4) cells transfected with 2 μg plasmid pEGFP-RNA2 (lane 1, lane 3) or pEGFP-C1 (lane 2 and 4) were harvested at 48 h posttransfection and assayed using antiprotein α sera by Western blot analysis.

RNA2 (Fig. 2A). In this construct, the expression of the EGFP-α fusion protein was under the control of a cytomegalovirus (CMV) promoter and the localization of protein α could be monitored by green fluorescence.

When the SB cells were transfected with pEGFP/RNA2, at 12 h posttransfection, the expression of EGFP-α fusion protein could be observed by fluorescence microscopy. As shown in Fig. 2B (image d), most green fluorescent signal is in the nucleus, especially enriched in the nucleolus. At 36 h posttransfection, EGFP-α fusion protein could be viewed in both nucleolus and cytoplasm (Fig. 2B, image g). DAPI, which can specifically bind to chromosome, was used to show the nuclear shape in blue color (Fig. 2B, images b, e, and h). The overlapping of green and blue fluorescent signal highlighted the localization of EGFP-α fusion protein in nuclear and nucleolus (Fig. 2B, images f and i). When SB cells were transfected with vector pEGFP-C1 and observed at 36 h posttransfection (Fig. 2B, image a), EGFP diffused throughout whole cell except the nucleolus.

To detect the subcellular localization of the fusion protein in mammalian cells, Cos-7 cells were also transfected with pEGFP-RNA2. At 12 h posttransfection (Fig. 2B, image D), EGFP-α fusion protein was seen concentrating in the nucleoli. At 36 h posttransfection, EGFP-α protein could be observed in the cytoplasm and nucleoli (Fig. 2B, image G). By contrast, EGFP alone was distributed diffusely throughout the cytoplasm and nucleoplasm with no specific pattern (Fig. 2B, image A). The localization of

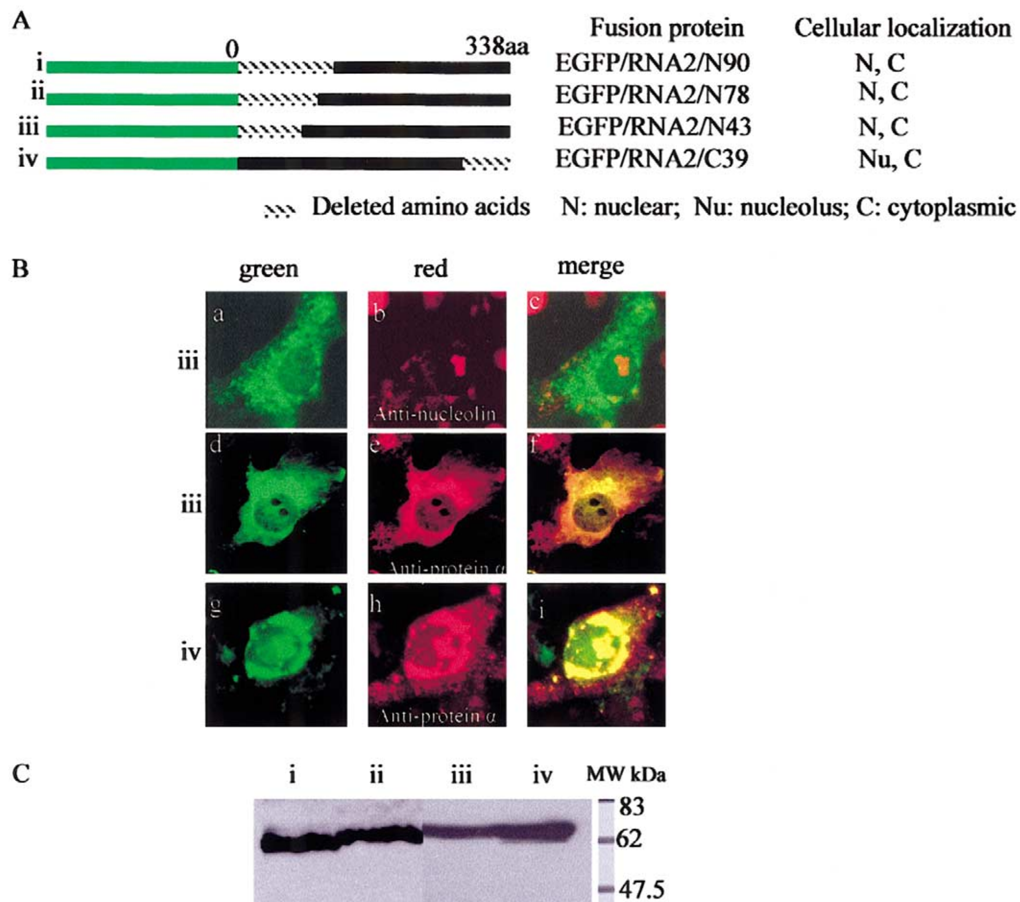

Fig. 3. (A) EGFP-α deletion mutations and their cellular localization. Green bar indicates EGFP and black bar indicates α deletion mutations. (B) Localization of EGFP-RNA2/N43 and EGFP-RNA2/C39 in Cos-7 cells. Cos-7 cells were transfected with pEGFP-RNA2/N43 and indirect IFA with antinucleolin mAb was carried out at 36 h posttransfection. Expression of EGFP-RNA2/N43 as a representative of EGFP-RNA2/N90 and EGFP-RNA2/N78 is shown in green (image a); the nucleolus is shown in red (image b), and merged image represents a digital superimposition of green and red signals, indicating the empty nucleolus localization of EGFP-RNA2/N43 (image c). Expression of EGFP-RNA2/N43 (image d) and EGFP-RNA2/C39 (image g) were detected by antiprotein α sera (images e and h) using indirect IFA at 36 h posttransfection. The merged image represents a digital superimposition of green and red signals, where areas of fluorescence colocalization are yellow-orange, indicating expression of fusion proteins (images f and i). (C) Western blot analysis with GFP antibody to detect the expression of EGFP fusion proteins. The whole-cell lysates of Cos-7 which were transfected with recombinant plasmids at 48 h posttransfection were separated on 12% SDS–PAGE and then analyzed by Western blot analysis with GFP antibody. Lane i: EGFP/RNA2/N90; lane ii: EGFP/RNA2/N78; lane iii: EGFP/RNA2/N43; lane iv: EGFP/RNA2/C39.

nucleolus was confirmed by using mAb against nucleolin with indirect immunofluorescence assay (Fig. 2B, images B, E, and H). The merged images (Fig. 2B, images C, F, and I) represent overlapping of green and red signals in yellow-orange. At 36 h posttransfection, equal aliquots of cell lysates from SB and Cos-7 cells transiently expressing EGFP-α fusion protein were analyzed by Western blot with antiprotein α antibody. A protein of the expected size of 66.3 kDa was detected in both SB (Fig. 2C, lane 1) and Cos-7 (Fig. 2C, lane 3) cells that were transfected with pEGFP-RNA2, but not in cells transfected with pEGFP-C1 (Fig. 2C, lane 2 and 4).

From the above result, it could be deduced that there are nucleolus localization signals in the protein α which can lead exogenous protein EGFP to the nucleolus of both fish and mammalian cells.

The nucleolus localization signal resides in the N-terminus of protein α

As protein α showed similar distribution in both cell types tested, experiments for the identification of the nucleolus localization signal were performed in Cos-7 cells only. Four N-terminal and C-terminal truncated protein α were constructed and fused with EGFP (Fig. 3A): pEGFP-RNA2/N90 (deleted N-terminal amino acids 1–90), pEGFP-RNA2/N78 (deleted N-terminal amino acids 1–78), pEGFP-RNA2/N43 (deleted N-terminal amino acids 1–43), and pEGFP-RNA2/C39 (deleted C-terminal amino acids 1–39). These recombinant plasmids were transfected to Cos-7 cells and viewed by confocal microscopy at 36 h posttransfection. EGFP/RNA2/N90 (data not shown), EGFP/RNA2/N78 (data not shown), and EGFP/RNA2/N43 (Fig. 3B, image a)

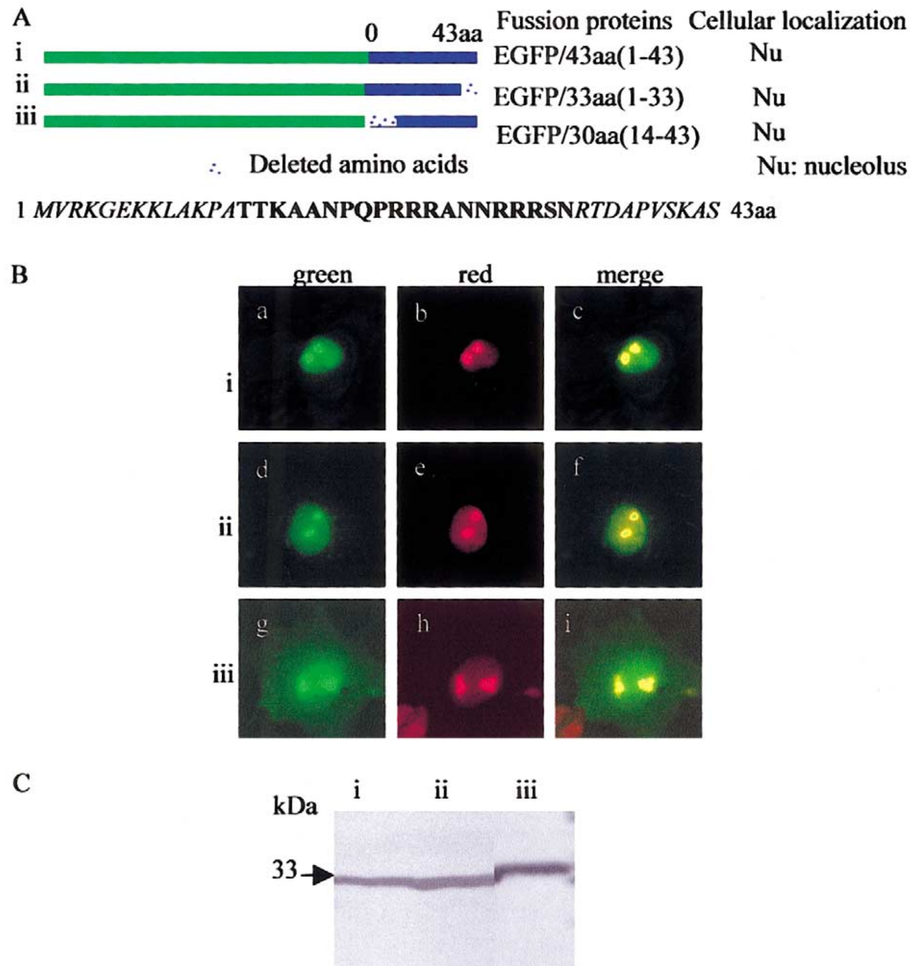

Fig. 4. (A) EGFP-N terminal amino acids of α fusion proteins, their cellular localization, and amino acid residues. Green bar indicates EGFP and blue bar indicates N-terminal amino acids of protein α. (B) The localization of EGFP-N terminal fusion proteins in Cos-7 cells. Cos-7 cells were transfected with pEGFP/43AA (image a), pEGFP/33AA (image d), and pEGFP/30AA (image g) and indirect IFA with mAb antinucleolin was performed at 36 h posttransfection. EGFP fusion proteins (images a, d, and g, green), nucleoli (images b, e, and h), and merged signals (images e, f, and I) are shown. The merged image represents a digital superimposition of green and red signals, where areas of fluorescence colocalization are yellow-orange, indicating the nucleolar localization of these three proteins. (C) Western blot analysis with GFP antibody to detect the expression of EGFP fusion proteins. The whole-cell lysates of Cos-7 which were transfected with recombinant plasmids at 48 h posttransfection were separated on 12% SDS–PAGE and then analyzed by Western blot analysis with GFP antibody. Lane i: EGFP/30AA; lane ii: EGFP/33AA; lane iii: EGFP/43AA.

showed the same distribution in Cos-7 cells; that is, at 36 h posttransfection, most protein was distributed in the cytoplasm and only background signal was observed in the nucleoplasm, but was completely excluded from nucleoli. However, the expression image of EGFP/RNA2/C39 (Fig. 3B, image g) was the same as that of wild-type EGFP-α fusion protein. The indirect IFA with antiprotein α sera further demonstrated the localization of EGFP/RNA2/N43 and EGFP/RNA2/C39 in transfected Cos-7 cells. The merged image (Fig. 3B, image f) of EGFP/RNA2/N43 fusion protein (Fig. 3B, image d, green) and red image of antiprotein α sera (Fig. 3B, image e) showed empty nucleoli. However, in the Cos-7 cell transfected with pEGFP/RNA2/C39 (Fig. 3B, image g), the indirect IFA showed the expression of EGFP/RNA2/C39 in nucleolus and cytoplasm (Fig. 3D, images h and i).

The expression of EGFP/RNA2/N90, EGFP/RNA2/N78, EGFP/RNA2/N43, and EGFP/RNA2/C39 was confirmed by Western blot analysis with anti-GFP antibody which detected the expected proteins (Fig. 3C). Taken together, these results indicated that the nucleolus localization signal of protein α is located in its N-terminal 43 amino acids.

Defining the nucleolus localization domain of protein α

To identify the functional domain of protein α responsible for nucleolus localization, the N-terminal 43 amino acids of protein α were fused to EGFP (Fig. 4A) and transiently expressed in Cos-7 cells. The N-terminal 43 amino acids of protein α were able to lead EGFP into the nucleoli of Cos-7 cell (Fig. 4B). To further confirm the nucleolar localization of EGFP/43aa observed by confocal microscopy, Cos-7 cells expressing EGFP/43aa was fractionated into cytoplasmic and nuclear component and mAb

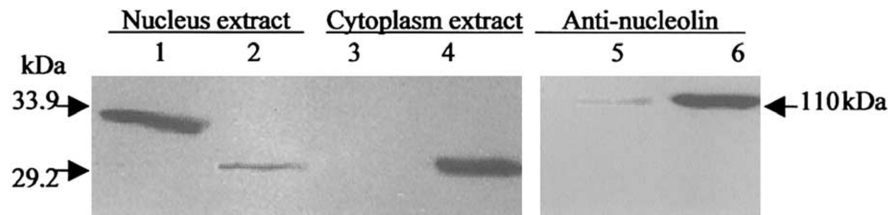

Fig. 5. Distribution of EGFP/43aa fusion proteins in Cos-7 cell subcellular fractions. Extracts of Cos-7 cells that expressed EGFP/43aa fusion protein were prepared and fractionated into nuclear (lane 1) and cytoplasmic (lane 3) fractions. A 10-μg sample of each fraction was separated on 12% SDS–PAGE and then analyzed by Western blot analysis with GFP antibody. Cos-7 cells expressing EGFP were used as control (lane 2 and 4). Antinucleolin mAb was used as a control to test the fidelity of the subcellular fractionations from cytoplasm (lane 5) and nuclear (lane 6).

against GFP was used in Western blot assay. EGFP-43aa fusion protein was not detected in the cytoplasmic fraction (Fig. 5, lane 3), whereas in the nuclear fraction, the EGFP-43aa fusion protein was in high concentration (Fig. 5, lane 1). However, EGFP as a control was found in both cytoplasmic (Fig. 5, lane 4) and nuclear fraction (Fig. 5, lane 2). There was more EGFP in cytoplasmic extract than in nuclear fraction. Antinucleolin mAb was used as a control to test the fidelity of the subcellular fractionations from nuclear (Fig. 5, lane 6) and cytoplasm (Fig. 5, lane 5). Combined with the results from confocal microscopy, we concluded that the 43 aa functioned as a nucleolus domain and could direct EGFP to nucleolus.

To narrow down the exact location of the localization signal, two additional deletion fragments from the N-terminus of proteins α were fused to EGFP (Fig. 4A) and transfected to Cos-7 cells. At 36 h posttransfection, transfected Cos-7 cells were fixed and antinucleolin mAb was used to define the location of nucleoli. Both EGFP/33aa (1–33) (Fig. 4B, image d) and EGFP/30aa (14–43) (Fig. 4B, image g) were located in the nucleoli of Cos-7 cells. The EGFP fusion proteins could be recognized by anti-GFP polyclonal antibody with expected sizes that were 32.7 kDa EGFP/30aa, 32.8 kDa EGFP/33aa, and 33.5 kDa EGFP/43aa in lanes i, ii, and iii, respectively (Fig. 4C). Taken together, a putative domain of nucleolar localization of protein α was identified to be between amino acid residues 14 and 33 of protein α.

Identification of ^{23}RRRANNRRR^{31} as the nucleolus localization signal of protein α

We next examined the amino acids from 14 to 33 to find which amino acids were more important in the nucleolar localization. Amino acid sequence analyses revealed that the Arg-rich region between amino acids 23 and 31 of protein α resembles nucleolar localization signals (NoLS) described in several other viral proteins and cellular proteins, such as human immunodeficiency virus type 1 (HIV-1) Rev and Tat (Kubota et al., 1996; Siomi et al., 1990), human T lymphotropic virus type I (HTLV-I) Rex (Siomi et al., 1988), African swine fever virus (ASFV) I14L (Goatley et al., 1999), the MEQ protein of Marek's disease virus (MDV) (Liu et al., 1997), parathyroid hormone-related protein (PTHrP) (Henderson et al., 1995), death effector domain-containing DNA-binding protein (DEDD) (Stegh et al., 1998), and angiogenin (Lixin et al., 2001). Table 1 shows a comparison between the NoLS of these proteins and the Arg-rich domain at N terminal of protein α.

To determine whether the Arg-rich domain between amino acids 23 and 31 of protein α is required for nucleolar localization, three constructs encoding fusion proteins containing different deletion mutations were transfected to Cos-7 cells (Fig. 6A). It was found that at 12 h posttransfection, for all these constructs, most signal was diffused in nucleoplasm, but was completely excluded from the nucleoli (Fig. 6B, images a, d, and g). After 36 h transfection, the fluorescent signal was viewed in cytoplasm, but not in the nucleolus (Fig. 6B, images ai, di, and gi). These phenomena were consistent with our conjecture that the amino acid residues ^{23}RRRANNRRR^{31} of protein α played a vital role in the nucleolus targeting of protein α in transfected cells. When the amino acids ^{23}RRR^{25} or ^{29}RRR^{31} were truncated from protein α, the specific nucleolar localization of the EGFP-α fusion protein was abolished, indicating that both ^{23}RRR^{25} and ^{29}RRR^{31} were responsible for directing nucleolus localization, but individually incapable of localizing EGFP-α fusion proteins to the nucleoli.

Table 1
Nucleolar localization sequences in viral and cellular proteins

Proteins	Sequences
Protein α	**RRR**ANN**RRR**
MEQ	**RRRKR**N**R**DAA**RRRRRK**Q
Tat	G**RKKRR**Q**RR**AP
Rev	**R**QA**RR**N**RRRR**WE**R**Q**R**
Rex	P**K**T**RRR**P**RR**SQ**RKR**P
I14L	MS**RR**N**KR**S**RRRRKK**PLNTIQ
PTHrP	G**KKKK**G**K**PG**KRR**EQE**KKKRR**T
DEDD	**KR**PA**R**G**R**ATLGSQP**KRRK**SV
Angiogenin	IM**RRR**GL

Note. Sequences from MEQ (from MDV), Tat and Rev (from HIV), Rex (from HTLV-1), I14L (from ASFV), parathyroid hormone-related peptide (PTHrP), death effector domain-containing DNA-binding protein (DEDD), and aegiogenin, which are required for nucleolar localization, are compared with N-terminal domain of protein α. The basic residues are shown in bold.

Fig. 6. (A) EGFP-deletion mutations of protein α, their cellular localization, and amino acid residues. Green bar indicates EGFP; black bar indicates backbone of protein α, and pink color indicates the deleted amino acids. (B) Localization of EGFP-deletion mutations of protein α in Cos-7 cells. Cos-7 cells were transfected with pEGFP/RNA2/d1RRR (images a and ai), pEGFP/RNA2/d2RRR (images d and di), and pEGFP/RNA2/d9aa (images g and gi) and observed at 12 and 36 h posttransfection, respectively. Nucleoli are stained in red by antinucleolin mAb (images b, bi, e, ei, h, and hi). The merged images (images c, ci, f, fi, j, and ji) represent a digital superimposition of green and red signals, showing that these deletion mutations are not expressed in nucleoli.

Subcellular localization of deletion mutations of protein α in SB cells

As the data of identifying the nucleolus localization signal were based on the Cos-7 cells, it was necessary to investigate whether this nucleolus localization signal worked in betanodavirus GGNNV host fish cells. We transiently expressed those different deletion mutants which functioned in Cos-7 cells into SB cells. The distribution of EGFP fusion proteins showed a similar pattern, as we predicted. At 36 h posttransfection, EGFP-RNA2/N43 was distributed in the nucleoplasm and cytoplasm except the nucleolus (Fig. 7, image a) and EGFP/43aa was enriched in the nucleolus (Fig. 7, image d). Following the deletion amino acids of ^{23}RRR^{25} (Fig. 7, image g), ^{29}RRR^{31} (Fig. 7, image j), or ^{23}RRRANNRRR^{31} (Fig. 7, image m) from protein α, the localization of EGFP-α fusion protein disappeared from the nucleoli. In addition, DAPI staining was carried out to show the nuclear in blue (Fig. 7, images b, e, h, k, and n). These observations demonstrated that the nucleolus localization signal ^{23}RRRANNRRR^{31} identified in Cos-7 cells played the same important role in betanodavirus GGNNV host SB cells.

Discussion

Localization to the nucleolus has been a part of the strategy for viruses to control both host cell and virus subgenomic RNA translation. The coronavirus N protein which is located in the nucleolus disrupted host cell division (Wurm et al., 2001); the HIV regulating proteins Tat and Rev made use of nucleolus for the trafficking of HIV-1 RNA (Michenzi et al., 2000; Siomi et al., 1990). In addition, some cellular proteins, including Werner syndrome (WRN) protein (Marciniak et al., 1998), DEDD (Stegh et al., 1998), and PTHrP (Henderson et al., 1995), which localize in nucleoli, have been found to be involved in the apoptosis events and the regulation of proliferation and differentiation. In this study, we have demonstrated nucleolar localization of GGNNV protein α in infected SB cells by IFA using monospecific antibody against protein α. Subsequently, transient expression of protein α showed its nucleolar distribution in fish and mammalian cells. However, the role of nucleolus in GGNNV infection needs further study.

The stages of the cell cycle may modulate protein targeting of specific subcellular compartment (Hiscox et al., 2001). Nucleoli are present when cells are in interphase

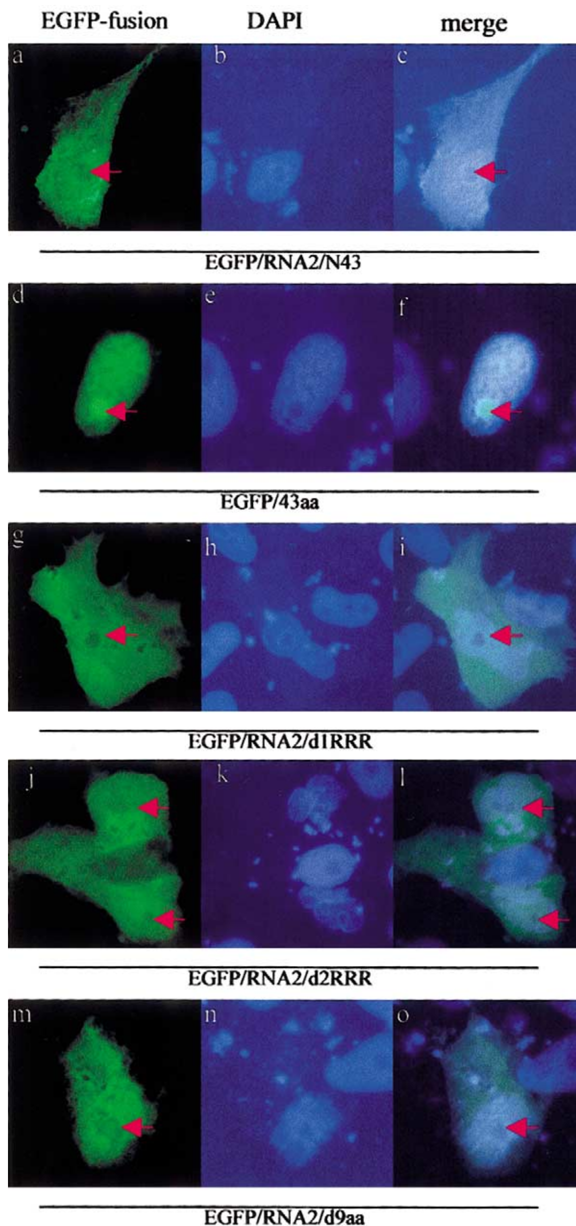

Fig. 7. Subcellular localization of deletion mutations of protein α in SB cells. SB cells were transfected with pEGFP/RNA2/N43 (image a), pEGFP/43aa (image d), pEGFP/RNA2/d1RRR (image g), pEGFP/RNA2/d2RRR (image j), and pEGFP/RNA2/d9aa (image m) and observed at 36 h posttransfection. SB cells expressing EGFP fusion proteins (green) and nuclear stained with DAPI (image b, e, h, k, n, and q, blue) are overlapped to form merged images c, f, i, l, and o, respectively. Red arrows indicate the location of nucleoli.

(Alberts et al., 1994). In the present study, at various time points, about 10% of infected or transfected cells exhibited nucleolus and cytoplasmic localization of GGNNV protein α. A possible reason for this observation could be that the fewer infected or transfected cells are at interphase, which showed localization of GGNNV protein α to the nucleolus, whereas other cells are in other phases of mitosis. Hiscox et al., (2001) also reported fewer cells with nucleolus localization of IBV N protein. However, mitosis alone cannot account for the absence of protein α from the nucleoli in the majority of infected or transfected cells, which suggests that localization of GGNNV protein α to the nucleoli might be dependent on particular events of the cycle that the infected or transfected cell is undergoing.

Protein import into the cell nucleus occurs through nuclear pore complexes either by diffusion or by signal-mediated transport. Proteins less than 40 kDa are able to enter the nucleus by passive diffusion; others generally require a specific nuclear localization signal (NLS), which is ATP dependent. NLSs have been identified for a number of viral and cellular proteins which have been classified as either monopartite or bipartite, depending on whether or not stretches of basic residues are interrupted by a 10 amino acid space region (Garcia-Bustos et al., 1991). Recently, several sequences have been defined as nucleolus localization signals. They consisted of clustered basic Arg/Lys residues and also functioned as nuclear transporting signals (Kubota et al., 1996; Siomi et al., 1988; Goatley et al., 1999; Zhu et al., 1999). In this study, EGFP was used to study the subcellular localization of protein α and search for its NoLS. EGFP-α fusion protein with a mass of 67 kDa could not be expected to diffuse into the nucleoplasm unless it contained an NLS/NoLS. The results of subcellular fraction analysis indicate that EGFP protein can diffuse to the nucleus, but its concentration in the nuclear extract is low. However, when EGFP was fused with N-terminal 43 aa of protein α, the fusion protein was mostly detected in the nuclear extract. So, we predicted that there must be a nucleolus localization signal in the 43 aa which contributes to the nucleolar localization of EGFP/43aa. There are two groups of basic amino acids in the N-terminal amino acids of protein α: ^{2}RKGEKKLAK^{10} and ^{23}RRRANNRRR^{31}, which resembled nucleolar localization signals that have been described in other viral and cellular proteins. The distribution of the deletion mutants of protein α in transfected cells demonstrated that ^{23}RRRANNRRR^{31} is a nucleolus localization signal. In this study, nucleolus localization signal of protein α was first identified in betanodaviruses, which may yield new insights into the mechanism of betanodaviruses infection. When ^{23}RRR^{25} or ^{29}RRR^{31} or ^{23}RRRANNRRR^{31} was deleted from protein α, EGFP fusion protein, which is too large (>40 kDa) to pass through the nuclear pore complexes by simple diffusion, was still observed in the nucleus. However, when the N-terminal 43 aa was deleted from protein α, EGFP/RNA2/N43 disappeared from the nucleus. These observations led to the possible suggestion that the protein α may contain other signals within 43 aa, such as ^{2}RKGEKKLAK^{10}, to be involved in its nuclear localization in the transfected and infected SB cells.

The mechanism of nucleolar localization of exogenous proteins depends on their nucleolar localization signals to bind either to an acid region in the nucleolus such as B23, or to rRNA, or to ribosome proteins (Li, 1997; Kubota et al.,

1999; Roller et al., 1996; Gorlich and Kutay, 1999). B23 may serve as a shuttle for the import of protein from the cytoplasm to the nucleolus coupled to the export of viral mRNA (Qiu and Brown, 1999). The nucleolin binding activity of hepatitis delta antigens (HDAgs) contributes to nucleolar localization and the modulation of the HDV replication (Lee et al., 1998). Many other RNA viruses make use of their RNA-binding proteins to localize into nucleolus to promote disassembly and assembly of the virus particle, such as the RNA-binding proteins in coronavirus (Hiscox et al., 2001), alphaviruses (Wengler, 1984), and alphanodaviruses (Hiscox and Ball, 1997). Nucleocapsids of Semliki Forest virus bind to 28srRNA to facilitate the release of the viral RNA genome (Rikkonen et al., 1992). Through the nucleoli of host cell, virus proteins appear to be involved in viral disassembly replication and assembly as well as in transcriptional and posttranscriptional regulation of gene expression. The nucleolus localization proteins ICP27 (Mears et al., 1995) and US11 (Roller et al., 1996) of HSV-1 are involved in posttranscriptional regulation of gene expression by binding ribosomal 60s subunit. Although the mechanism by which the betanodavirus protein α was transported to the nucleolus was not elucidated in this study, the nucleolar localization signal in betanodavirus protein α has been demonstrated. The expression of protein α was detected in both nucleolus and cytoplasm of transfected and infected cells. Based on this we speculate that by using the nucleolus localization signal ^{23}RRRANNRRR^{31}, protein α as the precursor of capsid protein could be imported to the nucleolus by a certain "carrier." Similar to nuclear import, export of a protein depends on the presence of a specific nuclear export signal (NES) (Gorlich and Kutay, 1999). These NES are short sequences characterized as being rich in leucine residues and have been identified in a variety of different proteins, the best study of which being the HIV Rev protein (Pollard and Malim, 1998). Protein α was detected in both nucleolus and cytoplasm. Possibly the regions other than 1–43 amino acids might be NES to be involved in its nuclear export, which could explain the observed cytoplasmic localization in this study. However, by analyzing amino acids of protein α, we were unable to definitely identify specific NES motifs in protein α. Two different possibilities exist that explain the failure in our identification of protein α NES. First, the NES has an unusual sequence that was not identified by initial sequence homology comparisons to known NES. Second, protein α may in fact lack a NES but is exported from the nucleus in complex with another NES-containing protein. Additional studies will be necessary to examine the mechanism of protein α shuttle between nucleolus and cytoplasm.

Some nucleolus localization proteins have been demonstrated to induce apoptosis. Localization of bax to nucleoli played a role in apoptosis-induction of glioma cells (Joy et al., 2000). DEDD was involved in apoptosis caused by CD95 (Stegh et al., 1998). In our previous work, protein α was found to induce apoptosis in transfected SB and Cos-7 cells. Although protein α failed to localize to the nucleolus when amino acids ^{23}RRRANNRRR^{31} were deleted, the apoptosis could not be prevented in transfected SB and Cos-7 cells (data not shown), indicating there is no relationship between nucleolus localization of protein α and its ability to induce apoptosis. Since protein α is located in both cytoplasm and nucleolus in transfected and infected cells, its cytoplasm localization might be involved in its apoptosis.

Materials and methods

Cells and viruses

Betanodaviruses (GGNNV) and SB cell line (Chong et al., 1987, Chong et al., 1990) were obtained from Agri-food and Veterinary Authority of Singapore (AVA). The SB cells were grown in modified Eagle's medium (MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.34% NaCl, 0.12% HEPES, and 2 mM glutamine at 23°C. GGNNV was originally isolated in 1992 from brain, head, kidney, and liver of greasy grouper *E. tauvina,* in Singapore. The SB cells were infected with the betanodaviruses GGNNV for propagation and inoculated cultures were harvested when 90% of cells in the monolayer showed specific CPE.

Cos-7 cells (a monkey kidney cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS and antibiotic (GIBCO) and grown at 37°C in 5% CO_2.

Antibodies

Polyclonal antiprotein α sera were produced by immunizing guinea pig with recombinant protein α expressed in *Escherichia coli*. The sera were then bled after boosting two times and purified by Column–protein A. The antibody was confirmed by Western blot analysis using GGNNV-infected SB cells and a specific 37-kDa protein could be detected (data not shown). Polyclonal anti-GFP sera was purchased from Molecular Probes (USA) and mAb anti-nucleolin was purchased from MBL (Japan).

Plasmids construction and cell transfection

Protein α encoding sequence and all deletion fragments were cloned into pcDNA3.1(+) vector (Clontech, USA) or into pEGFP-C1 vector (Clontech) in-frame with the EGFP and sequenced to verify reading frame. The primers and restriction enzymes site used to construct different recombinant plasmids were shown in Table 2.

The deletion mutants of pEGFP/RNA2/d1RRR, pEGFP/43AA/d2RRR, and pEGFP/43AA/d9aa acquired by using QuickChange Site-Directed Mutagenesis Kit (Stratagene) and primers are shown in Table 2. Briefly, recombinant

Table 2
Primers for constructing recombinant plasmids and deletion mutants

Plasmids' name	Forward primer	Reverse primer
PcDNA3.1/RNA2	GCGAATTCATGGTACGCAAAGGTGAGAAG (*Eco*RI)	GCTCTAGATTAGTTTCCCGAGTCAACCCTGG (*Xba*I)
pEGFP/RNA2	GCGAATTCTATGGTACGCAAAGGTGAGAAG (*Eco*RI)	GTGTCGACTTAGTTTCCCGAGTCAACCCT (*Sal*I)
pEGFP/RNA2/N90	GCGAATTCTCGACTGGGACACGCTGCTAGAATC (*Eco*RI)	GTGTCGACTTAGTTTCCCGAGTCAACCCT (*Sal*I)
pEGFP/RNA2/N78	GCGAATTCTGTTGTTGTTGACGCAACCATCGTC (*Eco*RI)	GTGTCGACTTAGTTTCCCGAGTCAACCCT (*Sal*I)
pEGFP/RNA2/N43	AGGAATTCCACTGTAACTGGATTCGGACGT (*Eco*RI)	GTGTCGACTTAGTTTCCCGAGTCAACCCT (*Sal*I)
pEGFP/43aa(1–43)	GCGAATTCTATGGTACGCAAAGGTGAGAAG (*Eco*RI)	GAGTCGACCGAGGCCTTAGACACAGGTGC (*Sal*I)
pEGFP/33aa(1–33)	GCGAATTCTATGGTACGCAAAGGTGAGAAG (*Eco*RI)	GCGTCGACATTACTACGCCGACGATTGTTA (*Sal*I)
pEGFP/30aa(14–43)	AGGAATCCCACCACCAAGGCCGCGAATCC (*Eco*RI)	GAGTCGACCGAGGCCTTAGACACAGGTGC (*Sal*I)
pEGFP/RNA2/d1RRR	CAAGGCCGCGAATCCGCAACCCGCTAACAATCGTCGGCGTAG	CTACGCCGACGATTGTTAGCGGGTTGCGGATTCGCGGCCTTG
pEGFP/RNA2/d2RRR	CCCCGCCGACGTGCTAACAATAGTAATCGCACTGACGCACCTG	CAGGTGCGTCAGTGCGATTACTATTGTTAGCACGTCGGCGGGG
pEGFP/RNA2/d9aa	CAAGGCCGCGAATCCGCAACCCAGTAATCGCACTGACGCACCTG	CAGGTGCGTCAGTGCGATTACTGGGTTGCGGATTCGCGGCCTTG

plasmid pEGFP/RNA2 was amplified by *pfuTurbo* DNA polymerase with designed primers and then digested with *Dpn*I restriction enzyme at 37°C for 1 h, followed by transformation with 1 μl reaction. The mutated plasmids were screened by sequencing with primer pEGFP: ACTACCT-GAGCACCCAGTCC.

Confluent monolayers (60–80%) of SB or Cos-7 cells grown on four-well chamber slides (IWAKI, Japan) were transfected with 0.5 μg (per chamber) of plasmid DNA purified by Qiagen plasmid Midi kits (USA) mixed with Lipofectamine plus reagent, according to the instructions of the manufacturer (Life Technologies, USA).

Indirect immunofluorescence assay and confocal microscopy

Transfected Cos-7 cells were cultured on four-well chamber slides (IWAKI). At 36 h posttransfection, cells were rinsed with phosphate-buffered saline and subjected to fixation with 4% paraformaldehyde for 25 min at room temperature and then permeabilized for 5 min in 0.2% Triton X-100 in PBS at room temperature for IFA. IFA was performed by incubating cells with primary antibodies (mAb antinucleolin or polyclonal antiprotein α sera) for 1 h at room temperature and then washed with phosphate-buffered saline with 0.05% Tween20 (PBST), followed by secondary antibodies conjugated with tetramethyl rhodamine isocyanate for 40 min at room temperature. Images were viewed and collected with a Zeiss confocal microscope LSM510.

DAPI staining

SB cells transfected with plasmids were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS as described above. Then cells were stained with DAPI (Molecular Probes) according to the manufacturer's instructions, viewed using a Leica 550 IW fluorescence microscope, and digital photographs were taken.

Subcellular fractionation

Cos-7 cells were cultured in 25 × 25 cm flasks and transfected with plasmid pEGFP-C1 or pEGFP/43AA. At 48 h posttransfection, cells were rinsed twice with ice-cold PBS and harvested by centrifuging at 2000 rpm for 5 min. The cell pellets were gently resuspended in hypotonic buffer [20 nM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.1 mM Na_3VO_3, 10% glycerol, 0.2% NP-40, 20 mM NaF, 1 mM DTT, 1 mM PMSF, complete proteinase inhibitors (Roche Molecular Biology): 1 tablet/8 ml]. The cell suspension was rocked at 4°C for 10 min and then centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant saved as the cytoplasmic extract. The nuclear pellet was washed in hypotonic buffer once and centrifuged as above. The crude nuclei was incubated with high salt buffer (hypotonic buffer without NP-40, 420 mM NaCl, 20% glycerol) and rocked at 4°C for 30 min. The lysate was then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant was collected as the nuclear extract.

Western blot analysis

Subcellular fractionation and extracts from SB or Cos-7 cells expressing EGFP or EGFP fusion proteins were separated by 12% SDS–PAGE and transferred to Hybond nitrocellulose membranes (Bio-Rad, USA). The membranes were blocked with 5% skimmed milk in PBST for 1 h at room temperature, washed with PBST once, and incubated with antibodies against GFP or protein α at room temperature for 1 h, then washed with PBST three times and incubated with secondary antibody conjugated with HRP at room temperature for 40 min. After rinsing three times, the specific proteins were visualized by enhanced chemiluminescence (Pierce, USA).

Acknowledgments

This work was supported by the Temasek Life Sciences Laboratory internal grant, Singapore. We acknowledge Dr.

David Balasandaram for helpful suggestion, and Dr. Orban Laszlo for critical reading of the manuscript.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roerts, K., Watson, J.D., 1994. "Molecular Biology of the Cell," 3rd ed. Garland Publishing, New York, pp. 381–382.

Ball, L.A., Hendry, D.A., Johnson, J.E., Rueckert, R.R., Scotti, P.D. (2000). Family *Nodaviridae,* in: van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B., (Eds.), "Virus Taxonomy" Seventh Report of the International Committee for the Taxomony of Viruses, Academic Press, San Diego, pp 747–755.

Ball, L.A., 1994. Nodavirus, in: Webster, R.G., Granoff, A. (Eds.), Encyclopaedia of Virology, Academic Press, London, pp. 919–925.

Carmo-Fonseca, M., Mendes-Soares, L., Campos, I., 2000. To be or not to be in the nucleolus. Nat. Cell. Biol. 2, E107–E112.

Chong, S.Y., Ngoh, G.H., Chew-Lim, M., 1990. Study of three tissue culture viral isolates from marine food fish. Singapore J. Primary Ind. 18, 54–57.

Chong, S.Y., Ngoh, G.H., Ng, M.K., Chu, K.T., 1987. Growth of lymphocystis virus in a sea bass, Lates calcarifer (Bloch) cell line. Singapore Vet. J. 11, 78–85.

Favre, D., Studer, E., Michel, M.R., 1994. Two nucleolar targeting signals present in the N-terminal part of Semliki Forest virus capsid protein. Arch. Virol. 137, 149–155.

Garcia-Bustos, J., Heitman, J., Hall, M.N., 1991. Nuclear protein localization. Biochim. Biophys. Acta 1071, 83–101.

Goatley, L.C., Marron, M.B., Jacobs, S.C., Hammond, J.M., Miskin, J.E., Abrams, C.C., Smith, G.L., Dixon, L.K., 1999. Nuclear and nucleolar localization of an African swine fever virus protein, I14L, that is similar to the herpes simplex virus-encoded virulence factor ICP34.5. J. Gen. Virol. 80, 525–535.

Gorlich, D., Kutay, U., 1999. Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell. Dev. Biol. 15, 607–660.

Henderson, J.E., Amizuka, N., Warshawsky, H., Biasotto, D., Lanske, B.M., Goltzman, D., Karaplis, A.C., 1995. Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Mol. Cell Biol. 15, 4064–4075.

Hiscox, J.A., Ball, L.A., 1997. Cotranslational disassembly of flock house virus in a cell-free system. J. Virol. 71, 7974–7977.

Hiscox, J.A., Wurm, T., Wilson, L., Britton, P., Cavanagh, D., Brooks, G., 2001. The coronavirus infectious bronchitis virus nucleoprotein localizes to the nucleolus. J. Virol. 75, 506–512.

Huang, B., Tan, C., Chang, S.F., Munday, B., Mathew, J.A., Ngoh, G.H., Kwang, J., 2001. Detection of nodavirus in barramundi, *Lates calcarifer* (Bloch), using recombinant coat protein-based ELISA and RT-PCR. J. Fish Dis. 24, 135–141.

Joy, A., Panicker, S., Shapiro, J.R., 2000. Altered nuclear localization of bax protein in BCNU-resistant glioma cells. J. Neurooncol. 49, 117–129.

Kaffman, A., O'Shea, E.K., 1999. Regulation of nuclear localization: a key to a door. Annu. Rev. Cell Dev. Biol. 15, 291–339.

Kubota, S., Copeland, T.D., Pomerantz, R.J., 1999. Nuclear and nucleolar targeting of human ribosomal protein S25: Common features shared with HIV-1 regulatory proteins. Oncogene 18, 1503–1514.

Kubota, S., Duan, L., Furuta, R.A., Hatanaka, M., Pomerantz, R.J., 1996. Nuclear preservation and cytoplasmic degradation of human immunodeficiency virus type 1 Rev protein. J. Virol. 70, 1282–1287.

Lee, C.H., Chang, S.C., Chen, C.J., Chang, M.F., 1998. The nucleolin binding activity of hepatitis delta antigen is associated with nucleolus targeting. J. Biol. Chem. 273, 7650–7656.

Li, Y.P., 1997. Protein B23 is an important human factor for the nucleolar localization of the human immunodeficiency virus protein Tat. J. Virol. 71, 4098–4102.

Liu, J.L., Lee, L.F., Ye, Y., Qian, Z., Kung, H.J., 1997. Nucleolar and nuclear localization properties of a herpesvirus bZIP oncoprotein, MEQ. J. Virol. 71, 3188–3196.

Lixin, R., Efthymiadis, A., Henderson, B., Jans, D.A., 2001. Novel properties of the nucleolar targeting signal of human angiogenin. Biochem. Biophys. Res. Commun. 284, 185–193.

Marciniak, R.A., Lombard, D.B., Johnson, F.B., Guarente, L., 1998. Nucleolar localization of the Werner syndrome protein in human cells. Proc. Natl. Acad. Sci. USA 95, 6887–6892.

Mears, W.E., Lam, V., Rice, S.A., 1995. Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. J. Virol. 69, 935–947.

Michienzi, A., Cagnon, L., Bahner, I., Rossi, J.J., 2000. Ribozyme-mediated inhibition of HIV 1 suggests nucleolar trafficking of HIV-1 RNA. Proc. Natl. Acad. Sci. USA 97, 8955–8960.

Misteli, T., Spector, D.L., 1997. Applications of the green fluorescent protein in cell biology and biotechnology. Nat. Biotechnol. 15, 961–964.

Pollard, V.W., Malim, M.H., 1998. The HIV-1 Rev protein. Annu. Rev. Microbiol. 52, 491–532.

Pyper, J.M., Clements, J.E., Zink, M.C., 1998. The nucleolus is the site of Borna disease virus RNA transcription and replication. J. Virol. 72, 7697–7702.

Qiu, J., Brown, K.E., 1999. A 110-kDa nuclear shuttle protein, nucleolin, specifically binds to adeno-associated virus type 2 (AAV-2) capsid. Virology 257, 373–382.

Rikkonen, M., Peranen, J., Kaariainen, L., 1992. Nuclear and nucleolar targeting signals of Semliki Forest virus nonstructural protein nsP2. Virology 189, 462–473.

Roller, R.J., Monk, L.L., Stuart, D., Roizman, B., 1996. Structure and function in the herpes simplex virus 1 RNA-binding protein U(s)11: Mapping of the domain required for ribosomal and nucleolar association and RNA binding in vitro. J. Virol. 70, 2842–2851.

Rowland, R.R., Kervin, R., Kuckleburg, C., Sperlich, A., Benfield, D.A., 1999. The localization of porcine reproductive and respiratory syndrome virus nucleocapsid protein to the nucleolus of infected cells and identification of a potential nucleolar localization signal sequence. Virus Res. 64, 1–12.

Shih, K.N., Lo, S.J., 2001. The HDV large-delta antigen fused with GFP remains functional and provides for studying its dynamic distribution. Virology 285, 138–152.

Siomi, H., Shida, H., Maki, M., Hatanaka, M., 1990. Effects of a highly basic region of human immunodeficiency virus Tat protein on nucleolar localization. J. Virol. 64, 1803–1807.

Siomi, H., Shida, H., Nam, S.H., Nosaka, T., Maki, M., Hatanaka, M., 1988. Sequence requirements for nucleolar localization of human T cell leukemia virus type I pX protein, which regulates viral RNA processing. Cell 55, 197–209.

Stegh, A.H., Schickling, O., Ehret, A., Scaffidi, C., Peterhansel, C., Hofmann, T.G., Grummt, I., Krammer, P.H., Peter, M.E., 1998. DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. EMBO J. 17, 5974–5986.

Tan, C., Huang, B., Chang, S.F., Ngoh, G.H., Munday, B., Chen, S.C., Kwang, J., 2001. Determination of the complete nucleotide sequences of RNA1 and RNA2 from greasy grouper (Epinephelus tauvina) nervous necrosis virus, Singapore strain. J. Gen. Virol. 82, 647–653.

Wengler, G., 1984. Identification of a transfer of viral core protein to cellular ribosomes during the early stages of alphavirus infection. Virology 134, 435–442.

White, J., Stelzer, E., 1999. Photobleaching GFP reveals protein dynamics inside live cells. Trends Cell Biol. 9, 61–65.

Wurm, T., Chen, H., Hodgson, T., Britton, P., Brooks, G., Hiscox, J.A., 2001. Localization to the nucleolus is a common feature of coronavirus nucleoproteins, and the protein may disrupt host cell division. J. Virol. 75, 9345–9356.

Zhu, H.Y., Yamada, H., Jiang, Y.M., Yamada, M., Nishiyama, Y., 1999. Intracellular localization of the UL31 protein of herpes simplex virus type 2. Arch. Virol. 144, 1923–1935.