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Identification of the nuclear export signals that regulate the intracellular localization of the mouse CMP-sialic acid synthetase ☆

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Abstract

The CMP-sialic acid synthetase (CSS) catalyzes the activation of sialic acid (Sia) to CMP-Sia which is a donor substrate of sialyltransferases. The vertebrate CSSs are usually localized in nucleus due to the nuclear localization signal (NLS) on the molecule. In this study, we first point out that a small, but significant population of the mouse CMP-sialic acid synthetase (mCSS) is also present in cytoplasm, though mostly in nucleus. As a mechanism for the localization in cytoplasm, we first identified two nuclear export signals (NESs) in mCSS, based on the localization studies of the potential NES-deleted mCSS mutants as well as the potential NES-tagged eGFP proteins. These two NESs are conserved among mammalian and fish CSSs, but not present in the bacterial or insect CSS. These results suggest that the intracellular localization of vertebrate CSSs is regulated by not only the NLS, but also the NES sequences.

Keywords: Nuclear export signal; Nuclear localization signal; Nucleus; Cytoplasm; Mammal; CMP-sialic acid synthetase; CMP-*N*-acetylneuraminic acid synthetase; Sialic acid; CMP-sialic acid; Biosynthesis of sialic acid

Sialic acids (Sias) are a family of nine-carbon sugars with a carboxylate group at C1 position, and consist of more than 50 derivatives of 2-keto-3-deoxy-nononic acids [1,2]. There are three major Sia species that differ in the substituents at C5 position, i.e., *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (KDN: 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) [3]. Sia is an indispensable sugar in mouse because the Sia-deficient mice are lethal in embryos [4]. Sia is usually situated at the non-reducing termini of glycan chains of glycoproteins and glycolipids and plays important roles in cell–cell and cell–extracellular matrix interactions [1,2,5]. Sia residues are synthesized by sialyltransferases that transfer Sia to the non-reducing termini of acceptor glycan chains of glycoconjugates from a donor substrate CMP-Sia. Therefore, the biosynthesis of CMP-Sia is an important step for the synthesis of Sia residues, and is catalyzed by a CMP-Sia synthetase (CSS, EC2.7.7.43; for review see [6]). One of the most prominent features of the vertebrate CSSs is their localization in the nuclear compartment, whereas other nucleotide sugar synthetases are mostly localized in the cytoplasm [6]. The mouse, human, and rainbow trout CSSs have been recently cloned [7–9] and the nuclear localization signals (NLSs) responsible for the targeting to nucleus are identified in mouse CSS (mCSS) using a series of the recombinant CSS mutants [10]. These vertebrate CSSs have two potential NLSs. The second NLS from the N-terminus is functional in mouse CSS [10].

Abbreviations: CMP-Sia, cytidine 5′-monophosphate sialic acid; CSS, cytidine 5′-monophosphate sialic acid synthetase; eGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; NES, nuclear export signal; Sia, sialic acid; NPC, nuclear pore complexes.

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Interestingly, however, during the course of the nuclear localization study of mCSS, we often observed the transiently expressed mCSS in both nucleus and cytoplasm of mammalian cells, although most mCSS is present in nucleus. This observation led us to hypothesize that some exporting mechanism from nucleus to cytoplasm is also involved in regulation of the intracellular localization of mCSS. We thus sought to identify the nuclear export signal (NES) in vertebrate CSSs.

Materials and methods

Materials. Anti-Myc monoclonal antibody (mAb.) 9E10 recognizing the Myc epitope (EQKLISEEDLN) was kindly provided by Dr. Gerardy-Schahn (Medizinisches Hochschule Hannover, Germany) and prepared as previously described [11]. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was purchased from Seikagaku Corp. (Tokyo, Japan).

Expression Plasmids. The eukaryotic expression plasmid pcDNA3-Myc-mCSS was kindly provided by Drs. Münster-Kühnel and Gerardy-Schahn (Medizinisches Hochschule Hannover, Germany). The pcDNA3-mCSS-Myc were prepared using pcDNA3-Myc-V5 vector which was kindly provided by Dr. Tiralongo (Griffith Univ., Gold Coast, Australia). The cDNAs encoding mCSS were amplified by PCR using the sense and antisense primers: 5′-AAT*GGTACC*GCCACCATGGACGCGCTGGAGAAG-3′ and 5′-GC*GCGGCCGC*CTTTTTGGCATGAGTTATT-3′, and cloned into pcDNA3-Myc-V5 through the *Kpn*I and *Not*I sites (italicized). The pcDNA3-Myc-mCSS and pcDNA3-mCSS-Myc comprise the full-length wild type mouse CSS with N-terminal Myc-tag and C-terminal Myc-tag, respectively. The integrity of all N-terminally Myc-tagged mutants was confirmed by the DNA sequencing.

Deletion mutants were introduced as previously described [12]. The cDNAs encoding ΔNES1 mutant were amplified by the first PCR using the sense and antisense primers: 5′-AAACTCGAGATGGACGCGCTGGAG AAGGG-3′ and 5′-GGCGGCGCGCAG$^{\Downarrow}$GATGCCTTTGCT-3′; or 5′-GCAAAGGCATC$^{\Downarrow}$CTGCGCGCCGCCCTG-3′ and 5′-GC*TCTAGA*CT ATTTTTGGCATGAGTTATTAACTT-3′. The pcDNA3-Myc-mCSS was used as template. The arrows shown in the primer sequences indicate the nucleotide position where the deletion was introduced. The second PCR was amplified using the sense and antisense primers: 5′-AAACTCGAGATGGA CGCGCTGGAGAAGGG-3′ and 5′-GC*TCTAGA*CTATTTTTGGCAT GAGTTATTAACTT-3′ using the first PCR products as templates. The second PCR products were digested with *Xho*I and *Xba*I, and ligated into pcDNA3 (pmCSSΔNES1). The cDNA encoding ΔNES2 mutant was amplified by the first PCR using the sense and antisense primers: 5′-AAACTCGAGATGGACGCGCTGGAGAAGGG-3′ and 5′-TCTAG ACTATTTTTGGCATGAGTTATTAACTTT$^{\Downarrow}$ACCGCTGCATTTG-3′. The PCR product was cloned into pcDNA3 through the *Xho*I and *Xba*I sites (pmCSSΔNES2). The integrity of all N-terminally Myc-tagged mutants was confirmed by the DNA sequencing.

The plasmids for the eukaryotic expression of N-terminally and C-terminally extended eGFP fusion proteins were generated by use of expression vector peGFP-N3 or peGFP-C1 (Clontech Laboratories Inc., CA, USA), respectively. The sense and antisense oligonucleotides that encode NES1, NES1+, and NES2 (see Fig. 2) of mCSS and HIV-Rev NES were synthesized. Oligonucleotides were generated with overhanging sequences to allow for the directed cloning into the *Sal*I and *BamH*I sites of peGFP-N3 and into the *Bgl*II and *Eco*RI sites of peGFP-C1 as shown in Table 1. Matching oligonucleotide pairs were annealed and ligated into the *Sal*I and *BamH*I sites of peGFP-N3 or *Bgl*II and *Eco*RI sites of peGFP-C1.

Cell culture. Chinese hamster ovary CHO and human cervix cancer HeLa cell lines were cultured in α-MEM (Invirtogen Corp., CA) and mouse fibroblast NIH-3T3 cell line was cultured in Dulbecco's modified Eagle's medium (Sigma) containing 100 U/ml penicillin and 0.1 mg/ml streptomycin. All media were supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum. All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

Table 1
Oligonucleotide primers used to construct the plasmids for the expression of the eGFP fusion proteins

Plasmid	Amino acid sequence	Oligonucleotides (5′–3′)
peGFP-C1-Rev	P^{70}VPLQLPPLERLTLDCS^{86}	*GATCT*CCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTGTAGCTAGG
		*AATTCCTAGCTACAATCAAGAGTAAGTCTCTCAAGCGGTGGTAGCTGAAGAGGCACAGGA
peGFP-N3-NES1	I^{61}KRLAGVPL^{69}	*TCGACATGATCAAGCGCCTGGCGGGGGGTTCCGCTCG*
		GATCCGAGCGGAACCCCCGCCAGGCGCTTGATCATG
peGFP-N3-NES1+	P^{57}LKNIKRLAGVPLIGWV^{73}	*TCGACATGCCACTGAAGAACATCAAGCGCCTGGCGGGGGGTTCCGCTCATTGGCTGGGTCG*
		GATCCGACCCAGCCAATGAGCGGAACCCCCGCCAGGCGCTTGATGTTCTTCAGTGGCATG
peGFP-N3-NES2	G^{408}RGAIREFAEHIFLLIE^{424}	*TCGACATGGGCCGGGGAGCCATCCGCGAGTTTGCAGAGCACGTTTTCCTACTGATAGAAG*
		GATCCTTCTATCAGTAGGAAAATGTGCTCTGCAAACTCGCGGATGGCTCCCCGGCCCCATG

The *Bgl*II and *Eco*RI extensions for peGFP-C1 and the *Sal*I and *BamH*I extensions for peGFP-N3 are italicized.

Transfection and the immunofluorescence observations. The CHO cells (1×10^6) cultured on the coverslips were transfected with 20 μg of the plasmids peGFP-N3-NES1 by electroporation (voltage : 210 V, capacitor : 960 μF) using the Gene Pulser Xcell TM electroporation system (Bio-Rad, CA, USA). For the other plasmids, peGFP-N3-Rev, peGFP-N3-NES1+, peGFP-N3-NES2, pmCSSΔNES1, and pmCSSΔNES2, 1.8×10^5 cells were transfected with 1–1.8 μg of the plasmids using Fugene 6 (Roche). After 48 h, the cells were fixed in methanol at −20 °C for 5 min. The Myc epitope was detected by sequential incubation with anti-Myc mAb.9E10 (13 μg/ml) at 37 °C for 2 h and with FITC-conjugated anti-mouse IgG + IgM (diluted 1:500 in PBS) at room temperature for 30 min. Nuclei was stained with DAPI (1 μg/ml) at 37 °C for 15 min. Fluorescence was observed under an Olympus BX51 microscope.

Results

Evaluation of the localization of mCSS in mammalian cells

mCSS is known to reside in nucleus [6,10] due to the NLS on mCSS [10]. At first, to investigate the localization of mCSS in mouse, Chinese hamster, and human cells, mouse NIH-3T3, Chinese hamster CHO, and human HeLa cells were transfected with pcDNA3-Myc-mCSS encoding the N-terminally Myc-tagged mCSS. In these three cells, mCSS was mostly localized in nucleus (Fig. 1A, arrow),

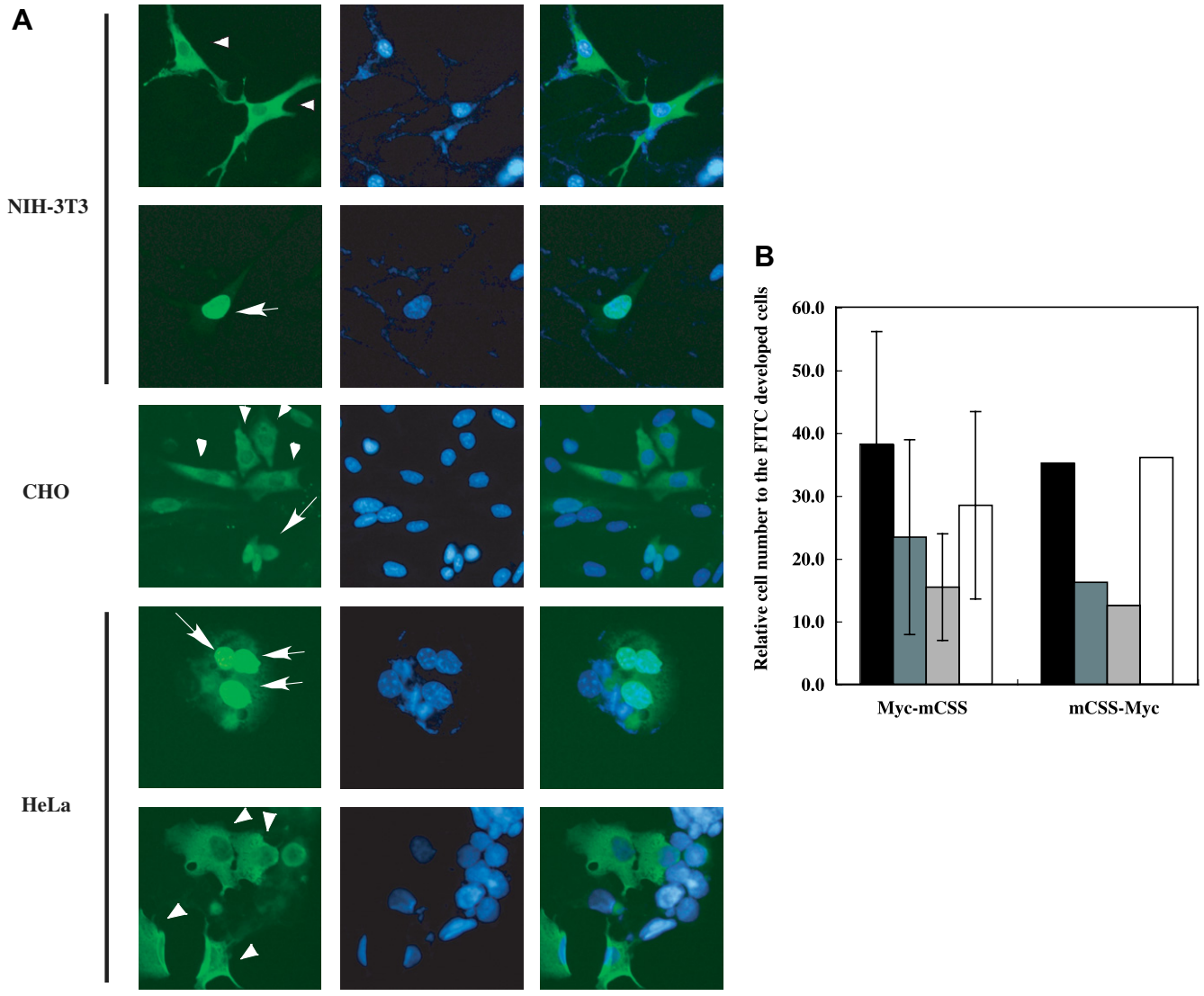

Fig. 1. Intracellular localization of mCSS. (A) Immunofluorescence and nuclear staining. NIH-3T3, CHO, and HeLa cells were transfected with pcDNA3-Myc-mCSS, and the transiently expressed N-terminally Myc-tagged mCSS was observed by immunofluorescence microscopy using anti-Myc mAb.9E10 and the FITC-conjugated anti-mouse IgG. Immunofluorescence and nuclear staining with DAPI of the same samples are shown at 400× magnification in the left and middle panels, respectively. The right panels show the merged images. The arrows and arrow heads indicate the cells containing the nucleus- and cytoplasm-localized mCSS, respectively. (B) The percentage proportions of the number of FITC-immunostained NIH-3T3 cells for each localization type of N- and C-terminal Myc-tagged mCSS, Myc-mCSS and mCSS-Myc. Closed square, type 1 (exclusive localization in nucleus); dark gray square, type 2 (localization in more nucleus than cytoplasm); gray square, type 3 (even localization in nuclear and cytoplasm); open square, type 4 (exclusive localization in cytoplasm). More than a hundred FITC-immunostained cells were counted. The experiments were performed in triplicate for pcDNA3-Myc-mCSS and once for pcDNA3-mCSS-Myc. Error bars indicate the SD.

but in some cells, mCSS is also observed in cytoplasm (Fig. 1A, arrowhead). The localization types could be classified into four groups: type 1, the exclusive localization in nucleus; type 2, the localization in both nucleus and cytoplasm, though predominant in nucleus; type 3, the even localization in nucleus and cytoplasm (entire cell); and type 4, the exclusive localization in cytoplasm. In general, type 1 and type 2 are recognized as the nuclear localization. To understand the localization of mCSS quantitatively, the cell number of each type of localization was counted in the mouse NIH-3T3 cells. As shown in Fig. 1B, the percentages for types 1 and 2 were 38% and 23%, whereas that for type 4 was 29%. This suggests that mCSS remains in cytoplasm in about one-third of the transfected cells. Similar results were also obtained with Chinese hamster CHO and human HeLa cells (data not shown). These results indicate that mCSS is localized in not only nucleus, but also cytoplasm in mammals such as mouse, Chinese hamster, and human.

To examine the effect of the N- and C-terminal tags on the mCSS molecule, the C-terminally Myc-tagged mCSS was subjected to the same experiments. As shown in Fig. 1B, the percentages for the localization types gave the same profiles between the N- and C-terminal Myc-tagged mCSSs, indicating that the localization patterns were not affected by the N- or C-terminal Myc-tag. Therefore, we used N-terminally Myc-tagged recombinant mCSS in the following experiments using mouse NIH-3T3 cells.

Search for the potential NES candidates in mCSS

Since the mCSS does not show the typical nuclear localization (types 1 and 2), but rather the type 3 and 4 localizations in 44% of the mCSS-expressing NIH-3T3 cells, the exporting mechanism from nucleus is suggested to operate concomitantly with the NLS. We thus sought to identify the NES in the mCSS molecule. NES is a transport signal that is necessary and sufficient to mediate nuclear export of large proteins [13]. The consensus short leucine-rich motif is deduced from the HIV REV and cellular protein kinase inhibitor proteins [14,15]: $LX_{2-3}LX_{2-3}LXL$, where X stands for any amino acid and L can be some other hydrophobic amino acids [16]. Based on the consensus sequence, two hydrophobic NES candidates were found in the N-terminal region (NES1, aa 61–69: IKRLAGVPL) and the C-terminal region (NES2, aa 408–424: GRGAIREFAEH IFLLIE) in mCSS (Fig. 2).

Localization of the mCSS mutants deleted in the potential NESs

To examine whether the NES1 and NES2 sequences are involved in the nuclear export of mCSS in mammalian cells, the localization of the mCSS deletion mutants devoid of the NES1 or NES2 sequence (ΔNES1 and ΔNES2) in mouse NIH-3T3 cells was determined by the indirect immunofluorescent microscopy. The immunostained cells were counted for each localization type. As shown in Fig. 3, the proportions of the type 1 and type 4 localizations were 54% and 17% for ΔNES1 and 71% and 16% for ΔNES2, whereas those values for the wild-type mCSS (Figs. 1B and 3A) were 38% and 29%. Compared with the wild-type mCSS, the nuclear localization was increased by 1.4- and 1.9-fold in ΔNES1 and ΔNES2, respectively. At the same time, the cytoplasmic localization was decreased by 41% in ΔNES1 and ΔNES2, respectively. The nucleocytoplasmic traffic is in balance with the strengths of the NLS and NES [16]. Therefore, the increased nuclear localization and the concomitant decreased cytoplasmic localization indicate that NES1 and NES2 usually work as NES in transport of mCSS from nucleus towards cytosol.

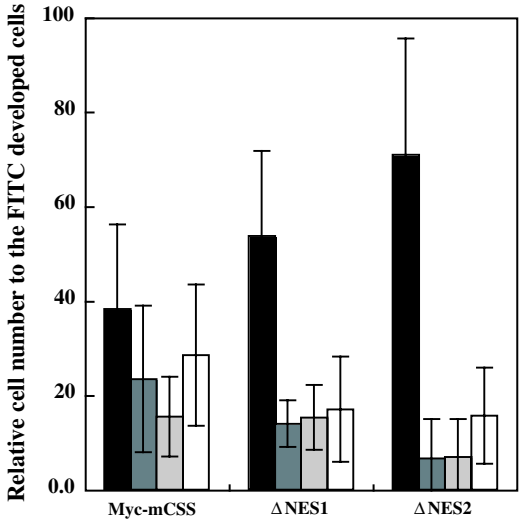

Fig. 3. Localization of the deletion mutants of mCSS in mouse NIH-3T3 cells. The percentage proportions of the number of FITC-immunostained cells for each localization type. Myc-mCSS, ΔNES1, and ΔNES2 stand for the cells transfected with pcDNA3-Myc-mCSS, pcDNA3-Myc-mCSSΔNES1 and pcDNA3-Myc-mCSSΔNES2. See the legend of Fig. 1B.

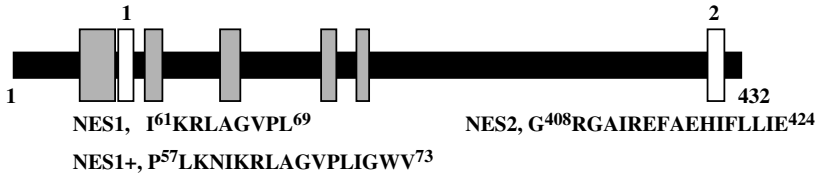

Fig. 2. A schematic representation of mCSS. The positions of the NES candidates are shown as open boxes with the corresponding amino acid sequences. The five amino acid sequence motifs conserved in bacterial and animal CSSs are boxed in gray.

The nuclear export activity of the potential NESs

To examine whether these potential NESs are functional in the nuclear export of proteins, the eGFP-protein fused with potential NESs were expressed in CHO cells, and the localization of eGFP-fusion proteins was observed by the direct fluorescence microscopy method. The eGFP protein itself showed the localization in more nucleus than cytoplasm, i.e., the type 2 localization (Fig. 4, eGFP), consistent with the previous observation [17]. As a positive control for the NES, HIV-Rev NES (aa 70–86, PVPLQLPPLERLTLDCS)-fused eGFP (eGFP-Rev) was expressed in CHO cells. As shown in Fig. 4 (eGFP-Rev), the eGFP-Rev protein was observed exclusively in cytoplasm outside the nucleus, indicating that the HIV-Rev NES peptide works as NES. Then, we analyzed the potential NESs for the nuclear export activity. The NES1-fused eGFP (eGFP-NES1) showed the type 2 localization (Fig. 4, eGFP-NES1) just like the eGFP protein (Fig. 4, eGFP). However, the extended form of NES1 (aa 57–73: PLKNIKRLAGVPLIGWV)-fused eGFP (eGFP-NES1+) protein showed the type 4 localization like the eGFP-Rev protein. These results indicate that NES1+ is functional in nuclear export of the eGFP protein, whereas NES1 itself

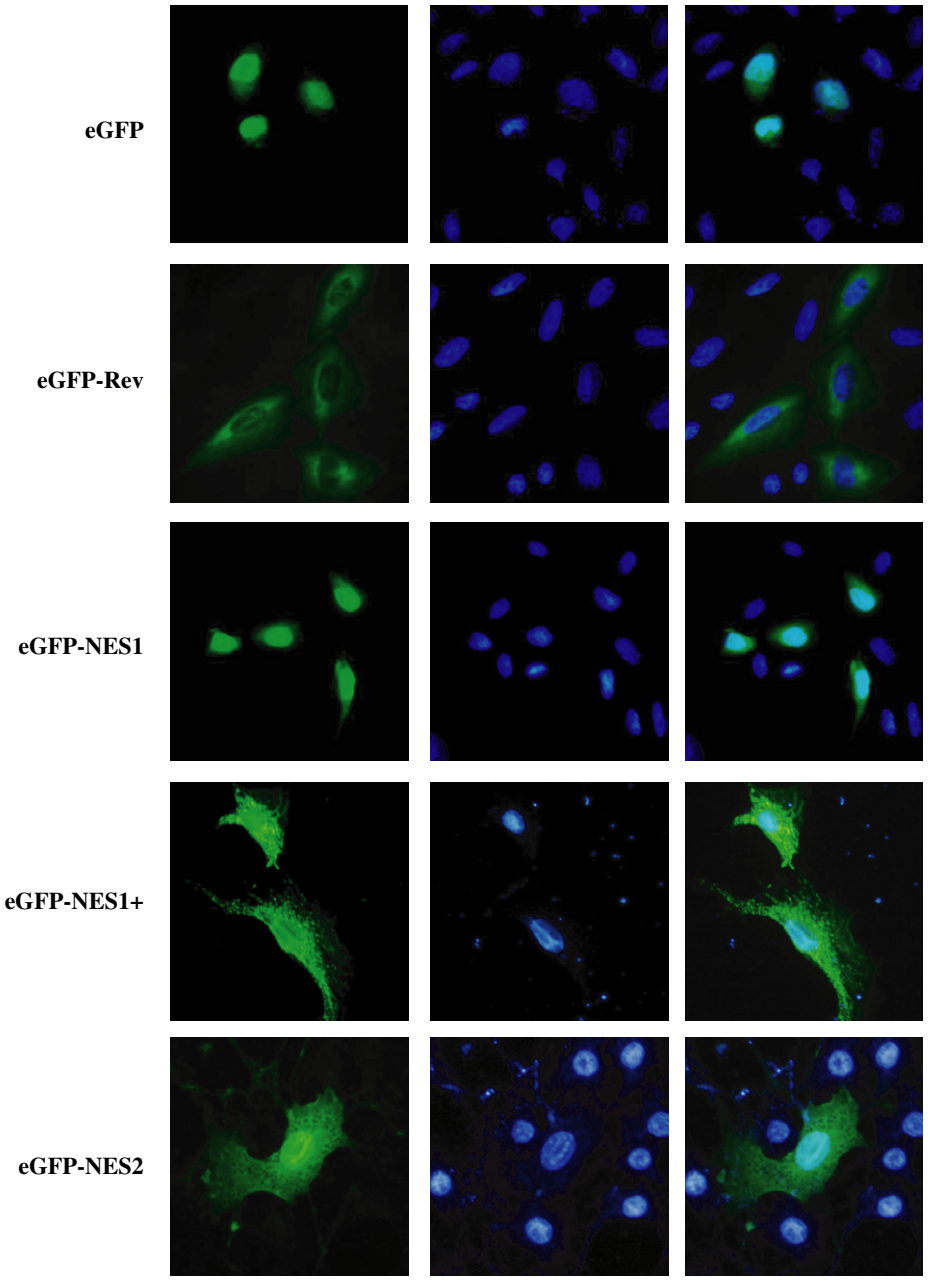

Fig. 4. Localization of the potential NES-tagged eGFP proteins in mouse NIH-3T3 cells. eGFP, eGFP-HIV REV, eGFP-NES1, eGFP-NES1+, and eGFP-NES2 were expressed in NIH-3T3 cells. Direct immunofluorescence and nuclear staining with DAPI of the same samples at 400× magnification are shown in the left and middle panels, respectively. The photos are merged in the right panel.

may be only a weak signal. Similarly, the NES2-fused eGFP (eGFP-NES2) protein showed the type 2 localization as is the case with the eGFP protein (Fig. 4, eGFP-NES2 and eGFP). Thus, the NES2 itself does not work as the nuclear export signal for the eGFP protein. All these results indicate that at least the NES1 peptide is functional as an NES of mCSS. However, it is difficult to conclude that NES2 is not a NES from these results, because it is known that the strength of NES activity of NES peptides depends on the structure and multiplicity of the peptides [16].

Discussion

The transport between nucleus and cytoplasm is through the nuclear pore complexes (NPC). Large-sized proteins of more than 40–60 kDa need nuclear transport signals to be transported through the NPC, whereas small proteins and substances can diffuse through them [13]. The NLS and NES sequences of transported proteins are involved in the nucleocytoplasmic transport [17,18], and the balance of the strength of these signals significantly affects the intracellular localization of nuclear proteins [16]. These peptide sequences are recognized by particular cellular receptors, such as importin family [19]. The cellular receptor for NES is Crm1p, a member of the karyopherin β family, which was first identified as CRM1/exportin-1 in fission yeast [20]. The consensus motif, which was first described for the HIV-1 Rev [14], is defined as a set of critically spaced hydrophobic residues with certain variations, like $LX_{2-3}LX_{2-3}LXL$ [16]. Many proteins owe their extranuclear distributions to NES, including human immunodeficiency virus Rev and Rex, protein kinase A inhibitor, Ran-binding protein 1, and MAPK kinase [21–24].

In this study, we found two potential NES sequences, NES1 and NES2, in mCSS deduced from the NES consensus motif, and identified these sequences as NES, based on the localization experiments of the deletion mutant deficient in the NES1 or NES2 sequence. However, the localization experiments of the NES1- or NES2-tagged eGFP protein suggest that the peptides themselves are weak in the nuclear export signals in the recombinant eGFP reporter protein. The strength of nuclear export signal activity of NES peptides themselves is demonstrated to differ depending on the primary sequence, the conformation in particular folding of proteins, and the number of the NES on the molecule [16,25]. As a clue to these possibilities, we showed that the extended form of NES1 (NES1+) worked as the NES of the eGFP-fusion protein. Further studies will have to be done to clarify how these two NES are functional in mCSS.

Interestingly, the NES sequences are conserved in vertebrate CSSs, except the frog (*Xenopus tropicalis*) CSS, as shown in Table 2. The consensus amino acid sequences for NES1 and NES2 conserved among the vertebrate CSSs are IKXLAGVPL and I(V,M)REFXEHIXL, where X is variable and the important hydrophobic amino acids are in bold. The frog and sea urchin (*Strongylocentrotus purpuratus*) CSSs contain homologous but incomplete NES1- and NES2-like sequences, and it remains to be clarified if they function as NES. Notably, no NES is found in the fruitfly (*Drosophila melanogaster*) CSS, consistent with the fact that it is localized in the Golgi, but not in nucleus [26] and that it contains no NLS (J. Tiralongo et al., to be published). In addition, no apparent NES sequences are found in any bacterial CSS (data not shown).

In this study, for the first time, we point out that the transport of mCSS between nucleus and cytoplasm is dynamic, because mCSS is not exclusively localized in nucleus as shown in Fig. 1. We also demonstrate that the dynamic nucleocytoplasmic transport of mCSS is at least due to the existence of NES in mCSS. The vertebrate CSSs have their enzymatic activity in the 270-amino-acid N-terminal domain. The remaining 160-amino-acid C-terminal domain has a similar alignment of the secondary structures for the HAD family phosphatase from *Haemophius influenzae* [27]. The function of the C-terminal domain remains to be clarified. However, considering that NES1 and NES2 exist in the N- and C-terminal domains allowing mCSS to be transported to cytoplasm, some cytoplasmic function of mCSS, especially the C-terminal domain, is implicated. In addition, the bacterial and insect CSSs contain no

Table 2
Comparison of the potential NES sequences in various animal CSSs

	NES1	NES2	Accession No.
Mus musculus	^{61}IKRLAGVPL	^{412}IREFAEHIFL	AJ006215
Homo sapiens	^{63}IKHLAGVPL	^{414}IREFAEHICL	AF271388
Gallus gallus	^{30}IKLLAGVPL	^{380}VREFAEHIFL	XP_416429
Oncorhynchus mykiss	^{50}IKVLAGVPL	^{404}VREFSEHILL	AB027414
Takifugu rubripes	^{48}IKMLAGVPL	^{404}MREFAEHIVL	AJ703819
Danio rerio	^{49}IKMLAGVPL	^{402}VREFAEHILL	AM262833
Xenopus tropicalis	^{28}IKDLAGRPL	^{378}IQEFAEYIEH	AM262832
Strongylocentrotus purpuratus	^{32}IKALAGQPL	^{383}GREFCDHLLL	AM262835
Drosophila melanogaster	None	None	BI609463

A multi-sequence alignment is performed with various animal CSSs to display maximum homology. The segments corresponding to the NES1 and NES2 of mCSS are shown. The highly conserved hydrophobic amino acid residues in the NES of various vertebrate nucleocytoplasmic proteins are in bold. The numbers indicate the first amino acid residues of the displayed sequences.

NLS [26] or NES (this study). Furthermore, the fruitfly CSS is targeted in the Golgi due to the hydrophobic stretch in the N-terminal region functional as a signal peptide [26]. These facts suggest that the CSS do not have to be destined to nucleus as far as the function of CSS is confined to the synthesis of CMP-Sia. To reveal the significance of the nucleocytoplasmic shuttling of the vertebrate CSS is an important on-going subject.

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