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Two Nuclear Export Signals Specify the Cytoplasmic Localization of Calcineurin B Homologous Protein 1

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We previously showed that calcineurin B homologous protein 1 (CHP1) interacts with nuclear apoptosis-inducing protein kinase DRAK2, and that overexpression of DRAK2 induces the nuclear accumulation of CHP1, although CHP1 usually resides in the cytoplasm [Matsumoto *et al.* (2001) *J. Biochem.* 130, 217–225]. Here we show that CHP1 has two functional nuclear export signal (NES) sequences in its carboxyl-terminal region. Treatment of several cell lines with leptomycin B, a specific inhibitor of CRM1-dependent nuclear export, induces the nuclear accumulation of CHP1. Moreover, CHP1-GFP fusion proteins with deletions or point mutations affecting the two putative NES sequences accumulate in the nucleus to a greater extent than wild-type CHP1-GFP. Tagging glutathione S-transferase-GFP fusion protein with each NES sequence caused a shift in their intracellular localization from all over the cells to the cytoplasm. These results suggest that after CHP1 has entered the nucleus, it is exported to the cytoplasm in an NES-dependent manner.

Key words: calcineurin B homologous protein, GST-GFP, intracellular distribution, Na^+/H^+ exchanger, nuclear export signal.

Abbreviations: CNA, calcineurin A; CNB, calcineurin B; CHP, calcineurin-homologous protein; DAPK, death-associated protein kinase; DRAK, DAP-kinase–related apoptosis-inducing protein kinase; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal.

CHP1 belongs to the superfamily of small calcium-binding proteins that contain an EF-hand motif, and shows extensive similarity to calcineurin B (CNB), a regulatory subunit of the cytosolic protein phosphatase calcineurin (*1*, *2*). Ubiquitously expressed CHP1 is modified by *N*-myristoylation and is thought to transduce Ca^{2+} signals to several effectors (*1*, *2*). In addition to the regulatory CNB subunit, calcineurin includes the catalytic subunit calcineurin A (CNA); maximal phosphatase activity is induced by the binding of Ca^{2+}/calmodulin to CNA (*3*, *4*). CHP1 also associates with CNA and is suggested to inhibit the phosphatase activity of calcineurin by altering interactions among CNA, CNB, and calmodulin (*5*). In addition to calcineurin, CHP1 binds to several other proteins and modulates their functions (*1*, *6–8*). Na^+/H^+ exchanger 1 (NHE1), a CHP1-interacting partner, is a cytoplasmic membrane protein that primarily maintains the intracellular pH and sodium concentration (*9*). The direct binding of CHP1 to the cytoplasmic tail domain of NHE1 regulates basal and serum-induced NHE1 exchange activity (*1*, *10*). CHP1 has also been identified as a factor required for the constitutive transport of vesicles to the apical plasma membrane (*2*), and it indirectly associates with the cytoskeleton via a putative cytosolic microtubule-binding factor (*6*). This association depends on the *N*-myristoylation of CHP1.

Recently, we reported that CHP1 interacts with KIF1Bβ2 and other members of the KIF1B family of microtubule-dependent motor proteins (*7*). KIF1Bβ2 is localized to synaptic vesicles in neurons, and its interaction with CHP1 is Ca^{2+}-dependent. Moreover, we have identified an apoptosis-inducing protein kinase, DRAK2, as a CHP1 binding protein (*8*). DRAK2 is a member of the death-associated protein kinase (DAPK) family, and its overexpression induces apoptosis-like cell death in NIH3T3 cells, similar to that observed for other DAPK family kinases (*8*, *11*). Most recently, we observed that CHP1 inhibits the kinase activity of DRAK2 and that this effect is Ca^{2+}-dependent (*11*). DRAK2 is localized in the nucleus of several cell lines in which it is expressed (*8*, *12*) because its kinase domain contains a typical lysine/arginine-rich nuclear localization signal (NLS) sequence, which is required for the active import of proteins into the nucleus. We previously showed that although CHP1 usually resides in the cytoplasm, especially in the perinuclear region, overexpression of DRAK2 induces its translocation into the nucleus, where it accumulates (*8*). This finding prompted us to examine how CHP1 returns to the cytoplasm: the possibility that this process is actively controlled had not been previously addressed. CHP1 and proteins that it interacts with are found at several subcellular locations, including the cytoplasmic membrane, the cytoplasm, the cytoskeleton, and the nucleus, and we were interested in determining whether this differential distribution depends on novel mechanisms. In this context, we analyzed putative signaling sequences in CHP1 to assess their role in nuclear export.

The nuclear export signal (NES) is a short sequence that has been identified as a motif required for the active nuclear export of proteins that reside in the cytoplasm regularly (*13*). The intracellular localization of many pro-

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teins, including cyclin B (*14*), an inhibitor of cAMP-dependent protein kinase (PKI) (*15*), and mitogen-activated protein kinase kinase (MAPKK) (*16*), is controlled by these signal sequences. The transport of these proteins between the nucleus and the cytoplasm has been shown to be inhibited by the antifungal agent leptomycin B (LMB). This antibiotic binds to CRM1, an NES receptor that mediates nuclear export via nuclear pore complexes (*17*, *18*).

In this study, we identify putative NES sequences in the carboxyl-terminal region of CHP1 and show that LMB treatment of cells induces the nuclear accumulation of CHP1. Moreover, derivatives of CHP1 with altered NES sequences accumulate in the nucleus to a greater extent than wild-type CHP1 does. Fusion proteins of glutathione S-transferase and green fluorescent protein (GST-GFP) tagged with each NES sequence show clear cytoplasmic localization, whereas GST-GFP without the NES is distributed all over the cells. These results suggest that the putative NES sequences are functional and that they regulate the cellular distribution of CHP1.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-7 cells, NIH3T3 cells and rat embryonic fibroblast EL2 cells (*19*) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. CHO cells were maintained in minimum essential medium-alpha modification medium supplemented with 10% FBS at 37°C in 5% CO_2.

Cells were transfected with expression vectors encoding CHP1 fusion proteins using cationic lipid reagent TransIT LT-1 (Mirus, WI) or FuGene6 (Roche Applied Science), according to the manufacturer's instructions.

Construction of Expression Vectors Encoding CHP Fusion Proteins—A rat CHP1 expression vector was constructed by integrating the CHP1 cDNA into plasmid pEF-BOS-EX (*20*), as described previously (*8*). For the construction of CHP1 with a C-terminal fusion of the green fluorescent protein (CHP1-GFP), the cDNA encoding the rat CHP1 open reading frame (0.6 kbp) was amplified by PCR using gene-specific primers incorporating restriction sites and then inserted into vector pEGFP-N (Clontech) in-frame with the GFP sequence.

cDNAs encoding CHP1 with C-terminal deletions (CHP1aa1-170, CHP1aa1-151, and CHP1aa1-134) were generated by PCR using reverse primers to delete the regions encoding the indicated CHP1 amino acids (171–196, 152–196, and 135–196, respectively). cDNAs encoding CHP1 alanine mutations (V138A/L139A, V143A/V145A/I147A, V179A/L180A, V183A/V185A, V138A/L139A+V179A/L180A, and V143A/V145A/I147A+V183A/V185A) were prepared by PCR-based mutagenesis (*21*) using primers with the corresponding mutations (Fig. 6A). Amplified cDNAs encoding the wild-type and mutant versions of CHP1 were cloned into expression vector pEGFP-N.

Expression vectors for GST-GFP fusion protein and its derivatives with NES1 or NES2 from CHP1 were constructed as follows. A PCR-amplified cDNA encoding GFP was integrated under the open reading frame of GST of pGEX4T-1 (Pharmacia), the plasmid constructed being named pGST-GFP. Oligonucleotides corresponding to the sequences of NES1 and NES2 (QVLRMMVGVNIS and EFVKVLEKVDVE, respectively) were synthesized with *Bam*HI-*Xho*I and *Hin*dIII-*Eco*RI sites at the 5′- and 3′-end, respectively. They were inserted in-frame into the site between GST and GFP in pGST-GFP. The open reading frames of GST-GFP, and derivatives with NES1 and NES2 were amplified by PCR, and then incorporated into the vector pcDNA3.1 (Invitrogene). The primary sequences of all constructs were confirmed by sequencing with automatic DNA sequencers (Applied Biosystems models 377 and 310).

GFP Fluorescence and Immunofluorescence Microscopy—Forty hours after transfection, cells were fixed for 10 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS). For observation of GFP fluorescence, fixed cells were stained with Hoechest 33342 to allow identification of the nucleus and then mounted with 50% glycerol/PBS (*22*). For immunostaining with affinity-purified anti-CHP antibodies, fixed cells were permeabilized by incubation in PBS containing 0.2% Triton X-100 for 10 min. After blocking with PBS containing 1% bovine serum albumin (BSA) (PBS/BSA) for 10 min, the cells were incubated with affinity purified anti-CHP antibodies in PBS/BSA for 1 h at room temperature and then washed with PBS. The cells were then incubated with FITC- or Alexa 488–conjugated anti-rabbit IgG (Molecular Probes) in PBS/BSA for 1 h, stained with Hoechst 33342 (0.1 μg/ml in PBS) for 10 min at 37°C (*8*), and washed with PBS and then with distilled water. Coverslips were mounted using 50% glycerol/PBS. Immunofluorescence was detected under a fluorescence microscope (Olympus, BX51) equipped with an ORCA-ER digital camera (Hamamatsu Photonics). Endogenous CHP1 was detected by basically the same protocol except that 4% goat serum was added to the blocking reagent.

Immunoblotting—Cells transfected with wild-type CHP1, mutant or GFP fusion proteins were homogenized in lysis buffer (20 mM Tris-HCl, pH7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 0.25M NaCl, 5 mM EDTA, and protease inhibitor cocktail (Roche Applied Science)) and then centrifuged at 15,000 ×*g* for 30 min at 4°C. The supernatant was used as the total protein lysate. Total cellular proteins (2 μg) were subjected to SDS-PAGE and the separated proteins were transferred to nylon membranes as described previously (*23*). Each membrane filter was incubated with anti-GFP serum (Clontech) or affinity purified anti-CHP1 antibodies (*8*) for detection of the transfected proteins. Immunoreactive materials were visualized by the ECL chemiluminesence method according to the manufacturer's instructions (Amersham Biosciences).

Homology Modeling—Molecular modeling of CHP1 was carried out using Swiss PdbViewer version 3.7 from GlaxoSmithKline. Since the rat CHP1 amino acid sequence was most closely related to that of the B subunit of human calcineurin, it was fitted to the X-ray diffraction coordinates for this protein (PDB 1M63) (*24*).

Materials—Anti-CHP1 antibodies were generated by immunizing a rabbit with bacterially expressed CHP1 tagged with hexahistidine, as described previously (*8*). Polyclonal antibodies were purified by affinity chromatography using purified CHP1 fused to glutathione S-

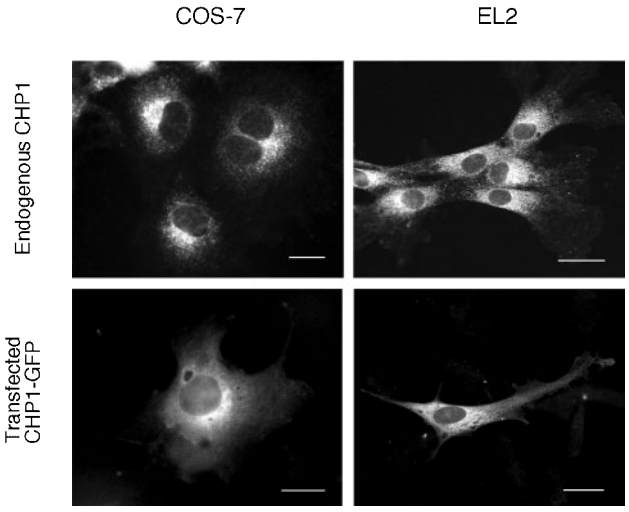

Fig. 1. **The subcellular distribution of endogenous CHP1 and GFP-tagged CHP1.** COS-7 cells and EL2 cells were cultured on poly-L-lysine coated coverslips. For GFP-tagged CHP1, the expression vector was transfected into the cells, and the cells were fixed 40 h later with 4% paraformaldehyde in PBS. For endogenous CHP1, untransfected cells were fixed and stained with anti-CHP1 antibodies as described under "MATERIALS AND METHODS." The subcellular distributions of GFP-tagged CHP1 and endogenous CHP1 were monitored by fluorescence microscopy. Representative results of three independent experiments are shown. The scale bar corresponds to 20 μm.

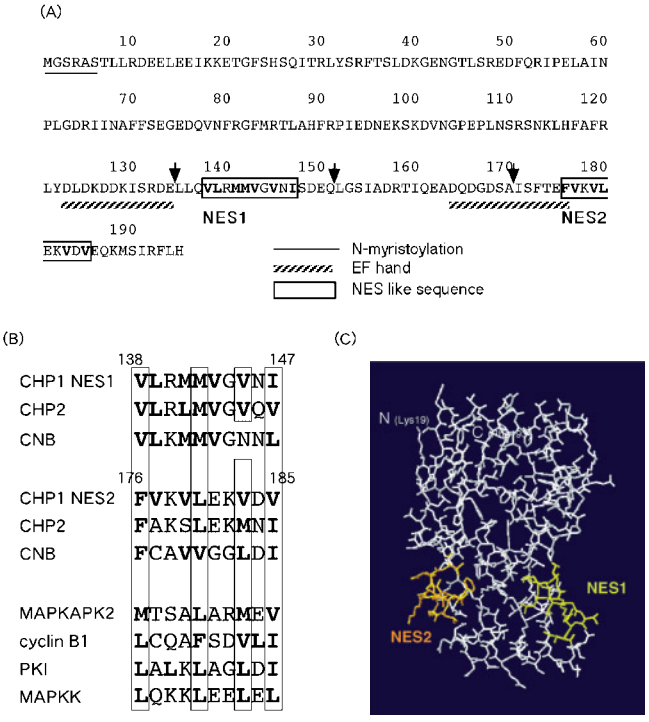

Fig. 2. **CHP1 has two putative NES sequences.** (A) Two putative NES motifs in the primary sequence of rat CHP1. NES1 and NES2 are boxed, and hydrophobic residues are indicated in bold. The *N*-myristoylation motif and the loop regions of two Ca^{2+}-binding EF-hand motifs are indicated by solid and hatched underlines, respectively. Arrows indicate the GFP fusion sites in the CHP1-GFP deletion mutants. (B) Comparison of putative NES sequences in CHP1 and other NES-containing proteins. The putative NES sequences of CHP1 (residues 138–147 and 176–185) and the corresponding sequences of CHP2 and CNB are aligned. For comparison, the NES sequences of MAPKAPK2 (*24*), cyclin B1 (*14*), PKI (*15*), and MAPKK (*16*) are also aligned with the established NES sequences. Important hydrophobic residues are boxed. (C) The putative NES motifs positioned on a model of CHP1 based on calcineurin B. The structure of CHP1 was predicted by homology modeling using the Swiss PdbViewer and crystallographic data for calcineurin B (*24*). NES1 and NES2, indicated in light and dark yellow, are on the surface of the molecule.

transferase as the ligand. The purified antibodies revealed a single band corresponding to the expected molecular size for CHP1 on a Western blot of extracts of several rat tissues (*8*), indicating that the antibodies used specifically react with CHP1. Leptomycin B (LMB) was kindly provided by Dr. M. Yoshida (Tokyo University). Stock solutions were prepared in absolute ethanol. KOD DNA polymerase (Toyobo) was used for PCR. Oligonucleotides were synthesized by Invitrogen. Other reagents and materials were of the highest grade commercially available.

RESULTS

Intracellular Distribution of Endogenous and GFP-Tagged CHP1 in Cultured Cells—Endogenous CHP1 is predominantly localized in the cytoplasm, with a higher concentration in the perinuclear region (Fig. 1), as previously described (*6*, *8*). Like endogenous CHP1, transfected CHP1 tagged with the green fluorescent protein (CHP1-GFP) (Fig. 1) and unmodified CHP1 (data not shown) are also found in the cytoplasm and the perinuclear region in several cell lines, including COS-7 cells and EL2 cells. Therefore, we used these expression systems to analyze the mechanism controlling the intracellular distribution of CHP1.

CHP1 Has Two Putative Nuclear Export Signals at Its Carboxyl Terminus—We searched for putative signaling sequences in the CHP1 primary sequence that may control its intracellular distribution (Fig. 2A). Besides a sequence required for *N*-myristoylation and two EF-hand motifs required for Ca^{2+} binding, we found two putative NES motifs (residues 138–147 and 176–185), designated as NES1 and NES2, in the carboxyl-terminal region of the protein (Fig. 2A, boxes). The CHP1 NES1 and NES2 elements are similar to typical NES sequences, which consist of leucine and other hydrophobic residues (isoleucine, valine, phenylalanine and methionine) with characteristic spacing (Fig. 2B). CHP1 exhibits high homology to tissue-specific isoform CHP2 (60% identity and 77% similarity) and calcineurin protein phosphatase regulatory subunit CNB (44% identity and 59% similarity) (*22*). Both putative NES motifs are conserved in CHP2 and one is also found in CNB (Fig. 2B). Of these three proteins, the X-ray structure has only been determined for CNB complexed with CNA (*24*). We predicted the three-dimensional structure of CHP1 by homology modeling, in which we fitted its primary amino acid sequence to coordinates obtained on crystallographic analysis of CNB. In this model, the two putative NES sequences of CHP1 are found on the hydrophilic surface of the molecule (Fig. 2C) at the same position as the corresponding NES sequence of the CNB-CNA holoenzyme (data not shown). There-

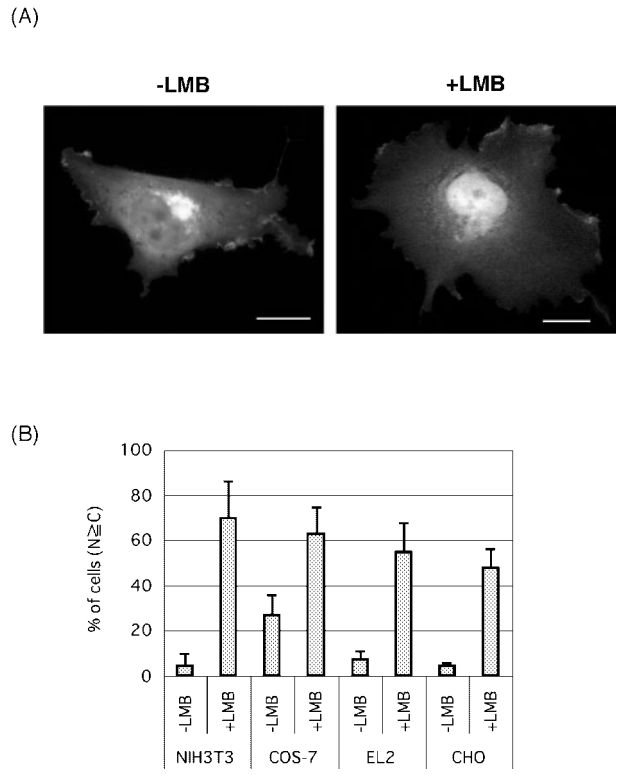

Fig. 3. **The effect of LMB on the nuclear accumulation of CHP1.** (A) A DNA construct encoding CHP1 was introduced into COS-7 cells. After 40 h, cells were treated with 10 ng/ml LMB for 1 h or untreated. The subcellular distribution of CHP1 was determined as described under "MATERIALS AND METHODS." Representative results of three independent experiments are shown; left, untreated control; right, LMB-treated sample. The scale bar corresponds to 20 μm. (B) NIH3T3, COS-7, EL2, and CHO cells were transfected with a DNA construct encoding CHP1. At 40 h post-transfection, the cells were treated with 10 ng/ml LMB for 1 h. The subcellular distributions of protein products were examined as described under "MATERIALS AND METHODS." The proportion of cells exhibiting a nuclear localization of CHP1 was determined by determining the fraction in which nuclear fluorescence corresponding to immunostained CHP1 was nearly equal to or stronger than cytoplasmic fluorescence (N ≥ C); the values for at least three experiments are plotted as means ± S.E. (>50 cells/experiment scored).

fore, we hypothesized that the putative NES sequences of CHP1 are of functional significance.

The Level of Nuclear CHP1 Increases in Response to LMB Treatment—To determine whether or not the intracellular localization of CHP1 depends on these NES motifs, we examined the effect of an inhibitor of NES-dependent nuclear export, LMB. A plasmid encoding CHP1 was transfected into several cell lines, and the distribution of the transiently expressed protein was determined by immunocytochemistry using an anti-CHP1 antibody (Fig. 3). In cells treated with the LMB solvent alone (ethanol), CHP1 was found in the cytoplasm, with specific enrichment in the perinuclear region (Fig. 3A, left panel). In contrast, in cells treated with LMB, CHP1 extensively accumulated in the nucleus (Fig. 3A, right panel). Figure 3B shows that the fraction of cells enriched with nuclear CHP1 increased following treatment with LMB for all cell lines examined.

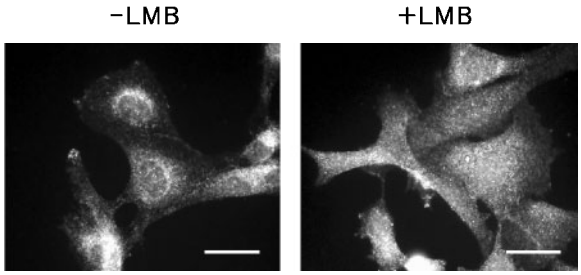

Fig. 4. **The effect of LMB on the intracellular distribution of endogenous CHP1 in EL2 cells.** EL2 cells were treated with 10 ng/ml LMB for 24 h or untreated. The subcellular distribution of CHP1 was determined as described under "MATERIALS AND METHODS." Representative results for a large number of cells examined in three independent experiments are shown; left, untreated control; right, LMB-treated sample. The scale bar corresponds to 20 μm.

To determine whether or not LMB treatment also causes endogenous CHP1 to accumulate in the nucleus, EL2 cells were cultured in medium containing LMB (10 ng/ml) for 24 h. The cells were fixed, and the intracellular distribution of endogenous CHP1 was visualized by immunostaining with an anti-CHP1 antibody. The level of endogenous CHP1 in the nucleus was also observed to increase after LMB treatment, although some CHP1 remained in the cytoplasm (Fig. 4). These results suggest that CHP1 is exported from the nucleus through an NES- and CRM1-dependent pathway.

The two NES Sequences Mediate the Cytoplasmic Localization of CHP1—In order to determine whether or not the putative carboxyl-terminal NES sequences are relevant to the nuclear export of CHP1, we examined the localization of several CHP1-GFP deletion mutants (Fig. 2A) in COS-7 cells. Two mutants with deletion of only NES2 (CHP1(aa1-170)-GFP and CHP1(aa1-151)-GFP) were distributed in a manner similar to that of wild-type CHP1-GFP (Fig. 5, upper right and lower left). In contrast, another mutant with deletion of both putative NES sequences (CHP1(aa1-134)-GFP) was predominantly found in the nucleus (Fig. 5, lower right). The mutant CHP1-GFPs were shown to be expressed in cells as proteins of the expected molecular sizes by SDS-PAGE and immunoblotting (data not shown). These results show that NES1 is required for the proper nuclear export of CHP1.

This analysis clearly showed that NES1 functions as a signal for the export of CHP1 from the nucleus. However, it remained possible that NES2 could function as an export signal and that either NES1 or NES2 could specify the localization of CHP1 when both are present. Therefore, we constructed and analyzed CHP1 derivatives containing site-specific mutations of NES1 or/and NES2 by replacement of hydrophobic and bulky residues (valine, leucine and isoleucine) with alanine residues (Fig. 6A). It has previously been reported that such substitutions render canonical NES sequences nonfunctional (*13*, *15*). The GFP-tagged NES mutant proteins were expressed in EL2 cells, and their distributions were monitored. Figure 6B shows the fraction of cells in which nuclear fluorescence due to GFP fusion proteins was nearly equal to or stronger than that of cytoplasmic fluorescence (N ≥ C). Wild-type CHP1-GFP and proteins with mutations in

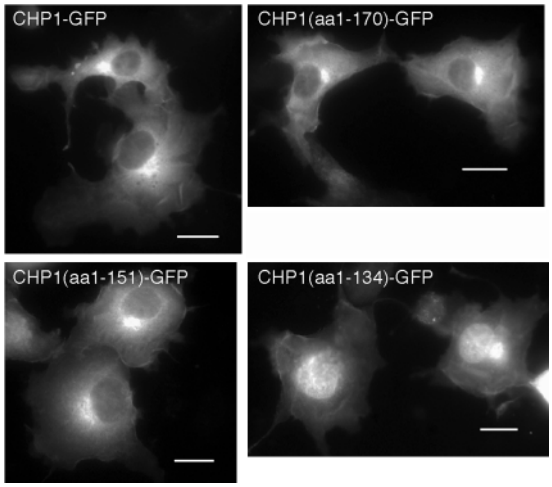

Fig. 5. **The subcellular distribution of NES deletion mutants of GFP-tagged CHP1.** Sequential deletions of the CHP1 carboxyl-terminal region containing NES sequences were made. Expression plasmids encoding wild-type CHP1 or deletion mutants fused with GFP were transfected into COS-7 cells. After 40 h, the cells were fixed with 4% paraformaldehyde in PBS. The subcellular distributions of protein products were determined by fluorescence microscopic observation. Representative images of a large number of cells in three independent experiments are presented. The scale bar corresponds to 20 μm.

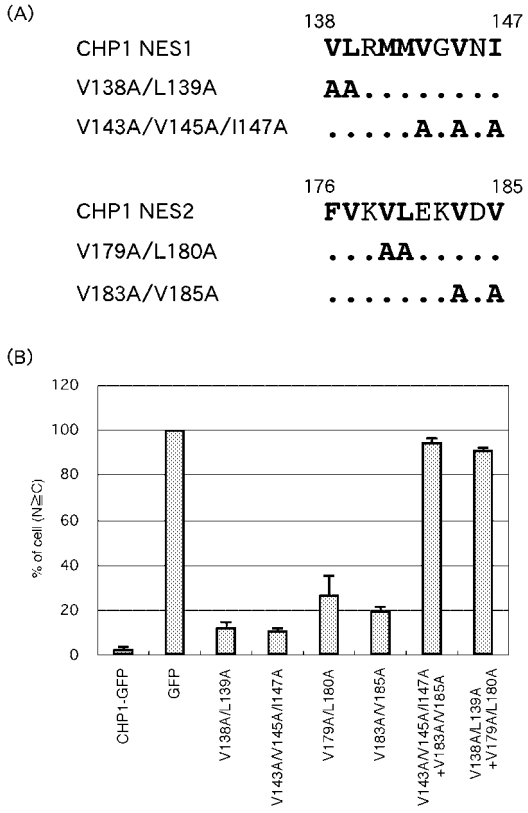

Fig. 6. **The subcellular distribution of GFP-tagged CHP1 derivatives with point mutations of the NES motifs.** (A) NES1 and NES2 sequences of the wild-type and mutant CHP1 derivatives. Two or three hydrophobic residues of each NES sequence were replaced by alanine, as indicated. (B) DNA constructs encoding GFP-tagged CHP1 with NES mutations were transfected into EL2 cells. The subcellular distributions of protein products were determined as described under "MATERIALS AND METHODS." The proportions of cells exhibiting a nuclear localization of the wild-type and mutant GFP-tagged CHP1 proteins were determined by determining the fraction in which nuclear fluorescence due to GFP was nearly equal to or stronger than cytoplasmic fluorescence (N ≥ C); the values for at least three experiments are plotted as means ± SE (>50 cells/experiment scored).

either NES1 or NES2 (V138A/L139A, V143A/V145A/I147A, V179A /L180A, and V183A/V185A) were predominantly located in the cytoplasm, while CHP1 derivatives with substitutions in both NES sequences (V138A/L139A + V179A/L180A and V143A/V145A/I147A + V183A/V185A) were found in the nucleus (Fig. 6B). These mutant proteins were shown to have the same molecular size as that calculated for the wild-type CHP1 on SDS-PAGE (data not shown). These results reveal that inactivation of either NES does not affect nuclear export. Therefore, we conclude that either NES1 or NES2 is enough for the proper localization of CHP1, and we suggest that NES2 also functions in the nuclear export of CHP1.

In order to determine whether or not NES1 and NES2 are sufficient to specify the cytoplasmic localization of a protein, GST-GFP fusion proteins tagged with each NES sequence (GST-NES1-GFP or GST-NES2-GFP) were expressed in COS-7 cells and then their intracellular distributions were observed (Fig. 7). GST-GFP without each NES was found uniformly in the nucleus and cytoplasm, while GST-NES1-GFP and GST-NES2-GFP were predominantly distributed in the cytoplasm. These results suggest that NES1 and NES2 themselves have sufficient nuclear export activity.

DISCUSSION

In this study, we found that CHP1 has two functional NES motifs in its carboxyl-terminal region and that the intracellular distribution of CHP1 is controlled by these sequences. Treatment of cells expressing exogenous or endogenous forms of CHP1 with LMB (Figs. 3 and 4) revealed that CHP1 is exported from the nucleus in a CRM1-dependent manner. Analyses of the effects of NES deletion and point mutations (Figs. 5 and 6) showed that both NES motifs are potentially functional and that at least one of them is required for the proper intracellular distribution of CHP1. Moreover, the results of experiments on GST-GFP fusion proteins with NES1 or NES2 suggest that the sequences are sufficient for localization of the protein in the cytoplasm (Fig. 7).

Canonical NES sequences, which have been found in typical nuclear-exported molecules such as Rev (*13*) and mitogen-activated protein kinase kinase (MAPKK) (*16*), have high leucine contents. Recent studies have shown that other hydrophobic residues, including isoleucine, valine, methionine and phenylalanine, are important for NES function (*14*, *25*). The two CHP1 NES sequences consist primarily of hydrophobic residues other than leucine, thereby indicating them to be low leucine-type NES motifs (*14*, *25*). The CHP1 NES sequence is also conserved in CHP2 and CNB (Fig. 2B). CHP2 has been reported to function as an activator for NHE like CHP1 (*26*). The intracellular distributions of CHP2 and CNB,

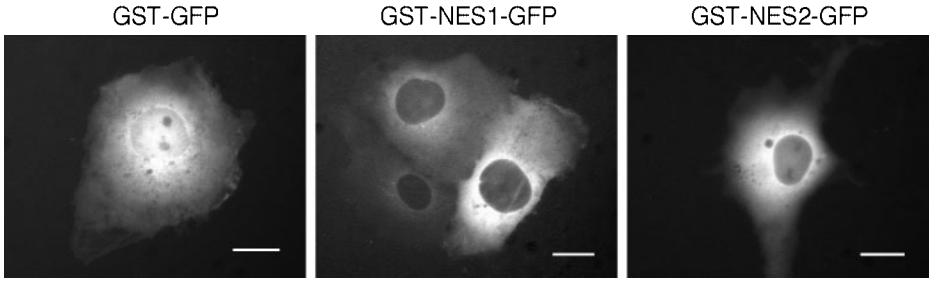

Fig. 7. **The subcellular distribution of GST-GFP fusion protein with or without the each NES sequence.** The expression vectors for GST-GFP and the derivatives with the NES1 or the NES2 were transfected into COS-7 cells, and 40 h after the transfection the subcellular distribution of the fusion proteins were observed by fluorescence microscopy. Representative images from a large number of cells in three independent experiments are presented. The scale bar corresponds to 20 μm.

like that of CHP1, might also be controlled by NES sequences. The CHP1 NES sequences identified in this study are located in the carboxyl-terminal α-helix of the EF-hands and the flanking region. Phospholipase C-δ1 also has a typical NES sequence in its EF-hand domain, which is located at a position similar to that of CHP1 (*27*). These observations imply that many other EF-hand proteins might also possess a functional NES.

The nuclear accumulation of both exogenous and endogenous CHP1 following LMB treatment is strong evidence that CHP1 is exported from the nucleus in an NES- and CRM1-dependent manner. However, endogenous CHP1 accumulated in the nucleus to a lesser extent than exogenous CHP1 did, and it also remained in the cytoplasm after treatment with LMB (Figs. 3 and 4). It is plausible that endogenous CHP1 is anchored to factors located in the cytoplasmic region, including the cytoskeleton (*6*) and vesicles that mediate transport to the plasma membrane (*1*, *2*), and a relatively small fraction is present in a free form. LMB treatment may cause this free CHP1 to be translocated to the nucleus.

Our finding that CHP1 is exported from the nucleus in an NES-dependent manner constitutes evidence for a mechanism that returns nuclear accumulated CHP1 to the cytoplasm. To our knowledge, this is the first report of the identification of functional NES motifs in a member of the superfamily of small Ca^{2+}-binding proteins, CHP1.

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