Nuclear-localized Calcineurin Homologous Protein CHP1 Interacts with Upstream Binding Factor and Inhibits Ribosomal RNA Synthesis

Maite Jiménez-Vidal, Jyoti Srivastava, Luanna K. Putney, and Diane L. Barber

From the Department of Cell and Tissue Biology, University of California, San Francisco, California 94143

Calcineurin homologous protein 1 (CHP1) is a widely expressed, 22-kDa myristoylated EF-hand Ca\(^{2+}\)-binding protein that shares a high degree of similarity with the regulatory B subunit of calcineurin (65%) and with calmodulin (59%). CHP1 localizes to the plasma membrane, the Golgi apparatus, and the nucleus and functions to regulate trafficking of early secretory vesicles, activation of T cells, and expression and transport of the Na-H exchanger NHE1. Although CHP1 contains nuclear export signals, whether its nuclear and cytoplasmic localization is regulated and has distinct functions remain unknown. We show that CHP1 is predominantly in the nucleus in quiescent fibroblasts, is translocated to cytoplasmic compartments with growth medium, and that translocation is inhibited by mutations in the nuclear export motifs. In a screen for proteins co-precipitating with CHP1 in quiescent cells we identified the upstream binding factor UBF, a DNA-binding protein and component of the RNA polymerase I complex regulating RNA synthesis. The CHP1-UBF interaction is restricted to the nucleus and inhibited by Ca\(^{2+}\). Nuclear retention of CHP1 attenuates the abundance of UBF in the nucleolus and inhibits RNA synthesis when quiescent cells are transferred to growth medium. These data show UBF as a newly identified CHP1-binding protein and regulation of RNA synthesis as a newly identified function for nuclear-localized CHP1, which is distinct from CHP1 functions in the cytosol.

A protein that localizes in multiple subcellular compartments often has spatially distinct functions. One example is the calcineurin B homologous protein CHP1, an N-myristoylated EF-hand Ca\(^{2+}\)-binding protein sharing ~40% identity with the regulatory B subunit of the protein phosphatase calcineurin and ~30% identity with calmodulin. CHP1, also known as p22, is evolutionarily conserved in human, rat, and Caenorhabditis elegans, is ubiquitously expressed in mammalian tissues, and localizes at the plasma membrane and in vesicular, cytosolic, and nuclear compartments. We previously identified CHP1 in a screen for proteins interacting with the C-terminal cytoplasmic domain of the plasma membrane Na-H exchanger NHE1 (1). Subsequent studies found that CHP1 is a cofactor essential for NHE1 activity (2–4) and also interacts with the Na-H exchanger isoform NHE3 (5). In vesicular compartments, CHP1 associates with membranes of the early secretory pathway (6) and is required for membrane traffic in a cell-free assay (7). In the cytoplasm, CHP1 complexes with microtubules (8) to facilitate microtubule-membrane interactions (9). Microtubule association can be mediated by direct binding of CHP1 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6), which associates with the microtubule cytoskeleton within the early secretory pathway, or to the kinesin-related motor KIF1Bβ (10), which mediates the transport of synaptic vesicles in neurons. In the cytosolic compartment CHP1 also directly binds the calcineurin catalytic A subunit and inhibits calcineurin phosphatase activity by preventing calmodulin binding to calcineurin A (11), likely because of sequence and structural similarities between CHP1 and calcineurin B (12).

In the nucleus, the function of CHP1 is less clear. CHP1 binds and inhibits the nuclear apoptosis-inducing protein kinase DRAK2 (13), although whether binding affects apoptosis remains undetermined. Nuclear accumulation of CHP1 is induced in cells overexpressing DRAK2 (14); however, the CHP1-DRAK2 association is not restricted to the nucleus but also is observed in the cytosol and Golgi apparatus (14). The carboxyl-terminal region of CHP1 includes two putative nuclear export signal (NES) sequences. NES motifs function in the active nuclear export of proteins that regularly reside in the cytoplasm. CHP1 accumulates in the nucleus upon treatment with leptomycin B (15), an inhibitor of CRM1-dependent nuclear export, and recombinant CHP1 with mutations in the NES sequences is localized predominantly in the nucleus (15). These findings confirm the functional significance of NES sequences in CHP1, and they suggest that CHP1 shuttles between cytoplasmic and nuclear compartments (15). Unresolved questions include whether CHP1 translocation between cellular compartments is determined by physiological signals and whether CHP1 has nucleus-specific functions.
The current study addresses both of these questions by showing that nuclear-localized CHP1 is regulated and has a distinct function compared with CHP1 in other cellular compartments. We found that endogenous CHP1 is predominantly restricted to the nucleus in quiescent fibroblasts but localizes to the Golgi apparatus and diffusely throughout the cytoplasm in fibroblasts maintained in growth medium. We also found that nuclear-localized CHP1 interacts with the upstream binding factors UBF1 and UBF2. UBFs are transcription factors that bind ribosomal DNA (rDNA) and form a preinitiation complex with RNA polymerase I to increase transcribed rDNA (16, 17). Our data indicate that nuclear-localized CHP1 associates with UBF and the promoter region of the rDNA genes and inhibits rRNA synthesis.

EXPERIMENTAL PROCEDURES

Materials—High purity trypsin was from Promega (Madison, WI). Fast-stain® (Coomassie Blue) was from Zoon Biotech (Shrewsbury, MA). a-Cyano-4-hydroxycinnamic acid was obtained from Hewlett-Packard. ZipTips were acquired from Millipore.

Cell Culture and DNA Transfections—CCL39 hamster lung fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g of glucose/ml and supplemented with 5% fetal bovine serum (FBS). Cells were transfected with the indicated plasmids by using the Lipofectamine Plus program (18).

Immunoprecipitation and Immunoblotting—Quiescent and FBS-stimulated CCL39 cells were lysed in modified radioimmune precipitation assay buffer (50 mm Tris-HCl, 135 mm NaCl, 3 mm KCl, 1% Nonidet P-40, protease inhibitors, 5 mm NaF, 10 mm sodium pyrophosphate, 1 mm glycerol phosphate, 1 mm sodium vanadate, pH 7.4) containing either 2 mm EGTA or 2 mm CaCl₂. Total cell lysates were used for immunoprecipitations, for subcellular fractionation, or directly for immunoblotting. To identify CHP1-interacting proteins, lysates were prepared from quiescent cells metabolically labeled for 18 h with [35S]methionine. For subcellular fractionation, total cell lysates were centrifuged to obtain a postnuclear supernatant and a pellet containing intact nuclei. Isolated nuclei were obtained by passing the postnuclear pellet over a 40% sucrose cushion, and a nuclear envelope fraction was obtained by lysing isolated nuclei in radioimmune precipitation assay buffer containing 250 mm NaCl and 0.5% Triton X-100 and retaining the pellet after centrifugation at 4,000 × g for 15 min. S100 soluble and P100 particulate fractions were obtained by centrifugation of the postnuclear supernatant at 100,000 × g for 20 min. For immunoprecipitations, total cell lysates were precleared for 60 min at 4 °C by incubating with protein G-Sepharose, incubated with antibodies to CHP1 that we generated or with polyclonal antibodies to UBF (Santa Cruz Biotechnologies, Santa Cruz, CA), and proteins in immune complexes were separated by SDS-PAGE. For immunoblotting, proteins were transferred to PDVF membranes, and membranes were probed with antibodies to CHP1, lamin, NHE1, and MEKK4 (Chemicon/Millipore), washed, and incubated with HRP-conjugated secondary anti-bodies. Bound antibody was visualized by chemiluminescence with an ECL detection kit (Amersham Biosciences).

Mass Spectrometry—The bands of interest were excised, macerated, and washed with 25 mm ammonium bicarbonate and 50% acetonitrile. A blank gel piece that did not stain with Fast-stain® was also processed in parallel as a negative control. After drying under reduced pressure in a SpeedVac concentrator (Savant, Holbrook, NY), the samples were placed in a solution of high purity trypsin (0.5 μg/μl of 25 mm ammonium bicarbonate). The proteolytic digestion was allowed to continue at 37 °C for 16 h. The resulting peptides were eluted from the gel with a solution of 50% acetonitrile and 5% trifluoroacetic acid in distilled water, and the volume was reduced in a SpeedVac concentrator. Samples were brought back to 15 μl with 1% trifluoroacetic acid and desalted with ZipTips according to the manufacturer’s instructions. Portions of unseparated tryptic digests were co-crystallized in a matrix of a-cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive Biosystems DE-STR MALDI time-of-flight mass spectrometer equipped with delayed extraction operated in the reflector mode.

Peptides were separated by HPLC prior to sequencing. The apparatus was fitted with a Michrom Bioresources MagicMS C18 column (0.2 × 50 mm; 5-μm particle size; 200-Å pore size), which was equilibrated with 7% acetonitrile and 0.1% trifluoroacetic acid in H₂O. A flow rate of 1 μl/min was established by using an Eldex Micropro pump. Peptides were eluted isocratically for 10 min followed by a linear gradient (0.95%/min) to a final mobile phase composition of 63% acetonitrile and 0.082% trifluoroacetic acid in H₂O. One to 2 μl HPLC fractions were spotted directly onto a MALDI target with 1.5 μl of a-cyano-4-hydroxycinnamic acid. Peptide sequence information was obtained by postsource decay sequencing as described previously (18). MS and postsource decay sequencing spectra were interpreted to yield protein identities using the MS-Fit and MS-Tag programs (18).

Immunolabeling and FUrđ Staining—CCL39 cells grown overnight on glass coverslips were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, permeabilized with methanol for 2 min, blocked with 3% BSA and 0.1% Triton X-100 for 30 min, and labeled with CHP1 or UBF antibodies. FUrđ incorporation was performed by incubating transfected cells with 2 μm FUrđ (Sigma) DMEM for 15 min at 37 °C and 5% CO₂ before fixing and permeabilizing as described above. FUrđ was visualized by incubating cells with monoclonal anti-FUrđ (Sigma). After incubating with fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, and Molecular Probes), cells were washed, mounted, and viewed using a Zeiss Axiopt fluorescence microscope. Confocal images were acquired using a Zeiss LSM 510 META with a 63 × oil immersion objective. The nuclear UBF signal was quantified using the ImageJ analysis program. The mean integrated density of UBF was measured from four regions of interest in the nucleoplasm and nucleolus (subtracting cytoplasmic background) in up to 20 cells from three separate experiments. Data were expressed as mean integrated density of UBF in the nucleolus/total (nucleoplasm + nucleolus).
CHP1 Interacts with UBF

Real Time PCR Quantification—RNA was extracted from cells using an RNAeasy kit (Qiagen, Valencia, CA), and cDNA was synthesized using iScript (Bio-Rad). The qPCR was run using PCR iQ SYBR Green Master Mix (Bio-Rad) using the Bio-Rad Chromo 4 real-time PCR detector. In each sample, both the GAPDH and 18S rDNA gene were amplified in triplicate. 18S gene expression was normalized to GAPDH. Hamster-specific primers used for GAPDH were: forward, 5-GTGAAGGTCCGGCGTGAACGGATT-3, and reverse, 5-CACAGTCTTCTGGGTGGCAGTGAT-3.

Chromatin Immunoprecipitation—CCL39 cells were cross-linked by incubation with 1% formaldehyde for 10 min at room temperature. Cells were swelled in hypotonic buffer (3 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% Nonidet P-40 and protease inhibitors), homogenized, and then nuclei were pelleted by centrifugation. Nuclei were lysed with nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and nuclear lysates were sonicated to generate 0.3–1.5-kbp chromatin fragments. After centrifugation, the supernatant was diluted 1:5 in dilution buffer (167 mM NaCl, 1.2 mM EDTA, 16.7 mM Tris–HCl, 0.01% SDS, 1.1% Triton X-100, pH 8.1) and precleared with protein A/G-Sepharose mixture that was pre-blocked with salmon sperm DNA.

For each immunoprecipitation, precleared nuclear lysates (equivalent to ~2 × 10⁶ cells) were incubated with antibodies to CHP1 or UBF or with IgG for 18 h at 4 °C, followed by incubation with blocked protein A/G-Sepharose mixture for 1 h. Beads were sequentially washed in low salt buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2% SDS, 0.5% Triton X-100, 150 mM NaCl), high salt buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2% SDS, 0.5% Triton X-100, 500 mM NaCl), LiCl wash buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% SDS, 0.5% Nonidet P-40), low salt buffer, and Tris-EDTA, pH 8.0. The bound DNA was then eluted, reverse cross-linked, and purified with QiaQuick PCR purification kit. Immunoprecipitated DNA was analyzed by PCR using primers specific for hamster ribosomal gene 5′-external transcriber spacer (ETS) sequence, and 18S and 28S coding sequences. qRT-PCRs were performed using an MJ Research® DNA Engine Opticon 2 system (Applied Biosystems). Reactions (250 μl) contained 12.5 μl of 2× IQ™ SYBR Green Supermix (Bio-Rad), 1.25 μl of immunoprecipitated DNA, and 250 nm primers. The cycling conditions were 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 84 °C for 10 s. The sequences of primers used in the reactions were 5′-ETS: 5-AAGACGTATGCCGTTACGG-3 and 5-AAGGCTGACCTGGCCGCTCGGG-3 (140-bp fragment, spanning from +960 to +1099); 18S coding region: 5-TCGCGGCAGCTGCTGTGCT-3 and 5-TCACAGTGTTATTGTC-3 (153-bp fragment); and 28S coding region: 5-ACAAGTACCGTAAGGGAAG-3 and 5-AACGGGGAGCGGGAAAGATC-3 (171-bp fragment).

RESULTS

Cytoplasmic CHP1 Translocates to the Nucleus in Quiescent Cells—In hamster lung CCL39 fibroblasts maintained in growth medium, immunolabeling of endogenous CHP1 shows predominantly extranuclear localization (Fig. 1A). Labeling is diffuse within the cytoplasm but strongest over the Golgi apparatus, consistent with CHP1 associating with membranes of the early secretory pathway (9) and regulating membrane trafficking (7). Although less strong, labeling is also seen along the plasma membrane, consistent with CHP1 being a co-factor for NHEs (2–5). However, in quiescent cells lacking FBS and growth factors for 18 h, immunolabeling is predominantly in the nucleus (Fig. 1A). A nuclear localization is consistent with CHP1 binding to DRAK2 (14), a nuclear protein. Moreover, these data show for the first time a physiologically regulated translocation of endogenous CHP1 between cytosolic and nuclear compartments.

We also used subcellular fractionation and immunoblotting to show regulated localization of endogenous CHP1. Lysates from quiescent CCL39 fibroblasts left untreated or treated for 4 h with medium containing 5% FBS were separated to obtain
isolated nuclei and S100 soluble and P100 particulate fractions. We verified the fractionation procedure by immunoblotting for the nuclear membrane protein lamin, the cytosolic MAP kinase MEKK4, and the plasma membrane NHE1 isofrom (Fig. 1B). Similar to our findings with immunolabeling, in lysates from quiescent cells the CHP1 signal is predominantly in the nuclear fraction (Fig. 1B, left). However, in lysates from cells treated with 5% FBS, the CHP1 signal is reduced in the nuclear fraction but strong in the P100 particulate fraction (Fig. 1B, right).

We next tested whether the regulated localization of CHP1 requires the previously identified NES sequences in the carboxyl-terminal region (15). For these studies cells transiently expressing recombinant GFP-tagged CHP1 wild-type (CHP1-WT), mutant CHP1 containing alanine substitutions in both NES sequences (V143A/V145A/I147A + V183A/V185A) (CHP1-NES), or GFP alone were maintained in growth medium for 24 h, transferred to serum-free medium for 18 h, and left untreated (quiescent) or treated with medium containing 5% FBS for an additional 4 h. In quiescent cells, CHP1-WT and CHP1-NES fluorescence is strongest in the nucleus. Fluorescence is also seen in the cytoplasm, likely because of overexpressed protein (Fig. 1C). In cells treated with FBS, CHP1-NES fluorescence is also predominantly nuclear and similar to the distribution in quiescent cells. However, CHP1-WT fluorescence is predominantly cytoplasmic and mostly excluded from the nucleus. Fluorescence of GFP alone is seen in both cytoplasmic and nuclear compartments in quiescent and FBS-treated cells. These data confirm nuclear localization of CHP1 in quiescent cells and show that NES sequence motifs are necessary for translocation of CHP1 from the nucleus to the cytoplasm with FBS.

**Nuclear CHP1 Interacts with UBF**—To begin testing whether nuclear CHP1 has functions distinct from those in the cytoplasm, we sought to identify proteins interacting with CHP1 in quiescent cells when CHP1 is predominantly restricted to the nucleus. Anti-CHP1 antibodies were used to immunoprecipitate endogenous CHP1 in lysates prepared from 35S-labeled CCL39 cells. Because CHP1 is a Ca2+-binding protein, we reasoned that Ca2+ might regulate the association of CHP1 with interacting proteins and used lysis buffer lacking or containing 2 mM CaCl2 for immunoprecipitations. Co-precipitating proteins separated by SDS-PAGE and visualized by autoradiography revealed several bands in the CHP1 but not control IgG immune complexes, including a band at 22 kDa that we predict is endogenous CHP1 (Fig. 2A). A doublet of proteins migrating at ~100 kDa consistently and robustly co-precipitated with CHP1 in the absence but not the presence of 2 mM CaCl2 and did not co-precipitate with IgG immune complexes. To determine the identity of these proteins, preparative immunoprecipitations were analyzed by matrix-assisted laser desorption ionizing-mass spectrometry (MALDI-MS) to obtain a peptide mass fingerprint. Peptide sequence was also analyzed by postsource mass spectrometry (MALDI-MS) to obtain a peptide mass fingerprint. Additionally, because immune complexes are enriched for CHP1, immunoblotting confirmed CHP1 is localized predominantly in the nucleus in quiescent cells but is also associated with the particulate fraction (Fig. 3E). These data indicate that in quiescent cells CHP1 interacts with UBF in the

**FIGURE 2. CHP1 Interacts with UBF in a Ca2+-sensitive manner.** A, autoradiograph of proteins co-precipitating in CHP1 or IgG immune complexes in lysates prepared in the absence or presence of Ca2+ from quiescent CCL39 fibroblasts labeled with [35S]methionine. Arrows indicate proteins of 97 and 94 kDa in CHP1 immune complexes in the absence but not presence of Ca2+ that are not in IgG immune complexes. MALDI-MS identified the 97-kDa protein as UBF1 and the 94-kDa protein as UBF2 (supplemental Fig. S1). B, immunoblot for UBF in CHP1 or IgG immune complexes isolated as described in A (upper panel) and in total cell lysate (lower panel). C, immunoprecipitation. C, immunoblot for UBF in cell lysates incubated with GST or GST and in total cell lysates (bottom panel). D, immunoblots for UBF and for CHP1 in UBF immune complexes isolated from cells maintained in the absence of FBS (∼) or for 4 h with FBS (+) (left) and in total cell lysate (right). E, immunoblots (IB) for UBF (upper panel) and for CHP1 (lower panel) prepared from CHP1 immune complexes isolated from quiescent cells.
nucleus in the absence but not the presence of Ca$^{2+}$. Whether CHP1 directly binds UBF remains uncertain because we were unable to show binding with bacterially expressed UBF and CHP. However, we also could not confirm UBF binding to an ERK1-positive control, perhaps because UBF was not correctly folded or posttranslationally modified.

**Nuclear Retention of CHP1 Inhibits rDNA Transcription**—Because UBF promotes transcription of rDNA genes we asked whether this is regulated by the nuclear localization of CHP1. In the nucleus, high levels of rDNA transcription occur within the nucleoli (19), which are readily visible by incorporation of FUr'd labeling and immunostaining with anti-FUr'd antibodies reveals significantly more intense Fur'd staining in cells treated with FBS compared with quiescent cells (Fig. 3, A and B). In control cells expressing GFP and in cells expressing recombinant GFP-tagged CHP1-WT (as described in Fig. 1C) there is a similar significant increase in Fur'd incorporation with FBS (Fig. 3C). However, in cells expressing GFP-tagged CHP1-NES, Fur'd labeling in quiescent cells is less than with CHP1-WT and does not increase with FBS (Fig. 3C), suggesting that nuclear retention of CHP1 inhibits FBS-stimulated rDNA transcription. We also used qPCR with primers for 18 S rRNA to quantify synthesis. Similar to our findings with Fur'd labeling, 18 S rRNA synthesis increased with FBS, as indicated in control cells expressing GFP alone (Fig. 3C). In quiescent cells, the abundance of 18 S rRNA with expression of CHP1-WT and CHP-NES was not different from control cells. However, with FBS treatment for 4 h, there was no increase with CHP-NES, and with CHP-WT the increase was markedly less than controls (Fig. 3C). Taken together, data from these two different approaches indicate that nuclear retention of CHP1 inhibits FBS-stimulated rDNA transcription.

**Nuclear CHP1 Binds the rDNA Promoter and Limits Abundance of UBF in the Nucleolus**—To understand how nuclear-localized CHP1 attenuates rRNA synthesis we first asked whether CHP1 associates with rDNA sequences. We used ChIP assays with antibodies to immunoprecipitate endogenous CHP1 and UBF in nuclear fractions of CCL39 fibroblasts. We included sets of primers specific for the 5′-ETS and the 18 S and 28 S coding regions of the rRNA genes. In agreement with previous findings that UBF binds to multiple sites distributed within the rDNA repeat (17), we found that UBF associates with all three regions of the hamster rRNA genes (Fig. 4A). CHP1 also associates with the rDNA gene promoter but is not detected at the coding regions (Fig. 4A). We then asked whether UBF or CHP1 binding to the rDNA promoter region is regulated. Quantitative real time PCR reveals that FBS induces a significant increase in UBF but not CHP1 (Fig. 4B), consistent with FBS translocation of CHP1 from the nucleus to the cytoplasm.

Several caveats regarding CHP1 binding to rDNA promoter prompted us to test additional mechanisms for how CHP1 might inhibit RNA synthesis. First is the relatively low abundance of CHP1 binding compared with UBF binding. Second is limited CHP1 immunolabeling seen in the nucleolus, where rDNA transcription occurs, compared with the nucleoplasm. Hence, we reasoned that another mechanism for inhibiting rRNA synthesis might be that CHP1 in the nucleoplasm sequesters UBF and limits UBF translocation to the nucleolus. In untransfected control cells (Fig. 5A) or GFP-expressing control cells (data not shown), immunolabeling for endogenous UBF confirms a predominant localization in the nucleolus with less protein in the nucleoplasm, as shown previously (19, 20). Quantifying fluorescence intensity reveals a significant increase in UBF labeling after 4 h with FBS compared with quiescent...
cells. A similar increase in nucleolar labeling with FBS is seen in cells expressing GFP-CHP1-WT, but there is no increase in cells expressing GFP-CHP1-NES (Fig. 5C). These data suggest that the CHP1-UBF interaction in the nucleoplasm may restrict UBF translocation to the nucleolus, which is required for efficient rDNA transcription.

**DISCUSSION**

CHP1 is the founding member of the calcineurin B homologous protein family that also includes CHP2 and CHP3 (CHP3 also known as tescalcin). CHP proteins are N-myristoylated and include EF-hand motifs that bind Ca\(^{2+}\) (1, 7) and NES sequences for nuclear export (15). CHP1 is ubiquitously expressed, but CHP2 is largely restricted to normal intestinal epithelia, although it is highly up-regulated in malignant cells (21). CHP3 is detected predominantly in adult mouse heart, brain, and stomach (22). CHP1 is a multifunctional protein, as evidenced by roles in promoting secretory vesicle trafficking (7), microtubule-membrane interactions (9), and NHE1 expression and activity (1–4), and in inhibiting calcineurin phosphatase activity (11). However, these functions of CHP1 occur in cytoplasmic-associated compartments, and whether the localization of CHP1 in the nucleus has distinct roles remains unknown.

Our study reveals several new findings on nuclear localized CHP1. First, we show that CHP1 localization is regulated by serum and likely growth factors. In growth medium CHP1 is in cytoplasmic compartments associated with the particulate but not soluble fraction of cell lysates. These data are consistent with CHP1 being myristoylated (7) and binding to microtubules (8), secretory vesicles (9), and NHE1 (1, 2), an integral plasma membrane protein. In quiescent cells, however, CHP1 translocates to the nucleus. Mutated NES sequences of CHP1 block translocation to the cytoplasm, resulting in nuclear retention with growth medium. Second, we show that nuclear CHP1 complexes with UBF, a transcription factor necessary for initiation of RNA transcription (23). Although we were unable to confirm whether CHP1 binds UBF directly, we showed that the association is blocked in the presence of Ca\(^{2+}\). Because CHP1 contains EF-hand motifs that bind Ca\(^{2+}\) (1), Ca\(^{2+}\)-binding might facilitate nuclear export of CHP1 or could occur after CHP1 is translocated to cytosolic compartments. Another possibility is that the association with UBF retains CHP1 in the nucleus in quiescent cells and with cell activation and increased intracellular Ca\(^{2+}\), Ca\(^{2+}\)-bound CHP1 dissociates from UBF and is exported from the nucleus. The regulated CHP1-UBF interaction is distinct from that shown for CHP1 binding to the nuclear kinase DRAK2 because overexpression of DRAK2 shifts the localization of CHP1 from the Golgi apparatus to the nucleus (13).

Additional new findings include that nuclear retention of CHP1 blocks increased RNA synthesis with growth medium and that CHP1 binds the rRNA gene promoter. Whether promoter binding contributes to CHP1 inhibiting RNA synthesis remains to be determined; however, our data suggest several possible mechanisms. Because CHP1 associates with the promoter but not the coding region of the rRNA genes, it might regulate the preinitiation complex but not transcription elongation. Another possibility is that association of CHP1 with the promoter impairs the ability of polymerase I to undergo transcription elongation. Because CHP1 does not contain a readily identified DNA-binding sequence, its association with the rRNA promoter likely depends on its interaction with transcription factors found only at the promoter. The ability of nuclear but not cytosolic CHP1 to associate with UBF and to inhibit rRNA transcription suggests but does not confirm that CHP1 might interfere with the assembly, function, or UBF binding of a preinitiation complex. Also consistent with our data is the possibility that release of CHP1 from the rRNA gene promoter permits transcription of rDNA.

Our finding that nuclear retention of CHP1 attenuates the abundance of UBF in the nucleolus suggests another mechanism for inhibition of rRNA synthesis by CHP1. UBF binding to the rRNA gene promoter is critical for the assembly of a productive preinitiation complex (24, 25). CHP1, independent of binding to the promoter region, could sequester UBF in the nucleoplasm and limit UBF translocation to the nucleolus. In two ChIP assays, however, expression of CHP1-NES did not significantly reduce the abundance of UBF binding to the promoter (data not shown). The activity of UBF is also modulated by phosphorylation (26–29), and an important question to answer is whether CHP1 binding affects UBF phosphorylation and possibly the association of UBF1 with a preinitiation protein complex.

These new data add UBF to an expanding list of proteins interacting with CHP1 and rRNA synthesis to an expanding list of cell processes regulated by CHP1. Future studies are needed to determine whether the association of CHP1 and UBF is direct, and whether CHP1 binding to the rDNA promoter is dependent on an association with UBF, and to resolve the regulatory mechanism whereby nuclear-localized CHP1 in quiescent cells is translocated to the cytoplasm in stimulated cells.
The emerging picture of CHP1 indicates that it is a multifunctional protein that regulates distinct cell processes in part by its localization in different cell compartments.

Acknowledgments—We thank Van Hoang and Susan Fisher for mass spectrometry analysis and Hiroshi Kanazawa for providing GFP-tagged CHP-WT and CHP-NES.

REFERENCES
1. Lin, X., and Barber, D. L. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 12631–12636
2. Pang, T., Su, X., Wakabayashi, S., and Shigekawa, M. (2001) J. Biol. Chem. 276, 17367–17372
3. Pang, T., Hisamitsu, T., Mori, H., Shigekawa, M., and Wakabayashi, S. (2004) Biochemistry 43, 3628–3636
4. Matsushita, M., Sano, Y., Yokoyama, S., Takai, T., Inoue, H., Mitsui, K., Todo, K., Ohmori, H., and Kanazawa, H. (2007) Am. J. Physiol. Cell Physiol. 293, C246–C254
5. Di Sole, F., Cerulli, R., Babich, V., Quiñones, H., Gisler, S. M., Biber, J., Murer, H., Burckhardt, G., Helme-Kolb, C., and Moe, O. W. (2004) J. Biol. Chem. 279, 2962–2974
6. Andrade, J., Pearce, S. T., Zhao, H., and Barroso, M. (2004) Biochem. J. 384, 327–336
7. Barroso, M. R., Bernd, K. K., DeWitt, N. D., Chang, A., Mills, K., and Szul, E. S. (1996) J. Biol. Chem. 271, 10183–10187
8. Timm, S., Titus, B., Bernd, K., and Barroso, M. (1999) Mol. Biol. Cell 10, 3473–3488
9. Andrade, J., Zhao, H., Titus, B., Timm Pearce, S., and Barroso, M. (2004) Mol. Biol. Cell 15, 481–496
10. Nakamura, N., Miyake, Y., Matsushita, M., Tanaka, S., Inoue, H., and Kanazawa, H. (2002) J. Biochem. 132, 483–491
11. Lin, X., Sikkink, R. A., Rusnak, F., and Barber, D. L. (1999) J. Biol. Chem. 274, 36125–36131
12. Naoe, Y., Arita, K., Hashimoto, H., Kanazawa, H., Sato, M., and Shimizu, T. (2005) J. Biol. Chem. 280, 32372–32378
13. Kuwahara, H., Kamei, I., Nakamura, N., Matsumoto, M., Inoue, H., and Kanazawa, H. (2003) J. Biochem. 134, 245–250
14. Matsumoto, M., Miyake, Y., Nagita, M., Inoue, H., Shitakubo, D., Take-moto, K., Ohtsuka, C., Murakami, H., Nakamura, N., and Kanazawa, H. (2001) J. Biochem. 130, 217–225
15. Nagita, M., Inoue, H., Nakamura, N., and Kanazawa, H. (2003) J. Biochem. 134, 919–925
16. Grummt, I. (1999) Prog. Nucleic Acids Res. Mol. Biol. 62, 109–154
17. Russell, J., and Zomerdijk, J. C. (2005) Trends Biochem. Sci. 30, 87–96
18. Clauser, K. R., Baker, P., and Burlingame, A. L. (1999) Anal. Chem. 71, 2871–2882
19. Grummt, I. (2003) Genes Dev. 17, 1691–1702
20. Jordan, P., Mannervik, M., Tora, L., and Carmo-Fonseca, M. (1996) J. Cell Biol. 133, 225–234
21. Jin, Q., Kong, B., Yang, X., Cui, B., Wei, Y., and Yang, Q. (2007) In Vivo 21, 593–598
22. Gutierrez-Ford, C., Levay, K., Gomes, A. V., Perera, E. M., Som, T., Kim, Y. M., Benovic, J. L., Berkovitz, G. D., and Slepak, V. Z. (2003) Biochemistry 42, 14553–14565
23. Comai, L. (2004) Adv. Protein Chem. 67, 123–155
24. Bell, P., Mais, C., Mccstay, B., and Scheer, U. (1997) J. Cell Sci. 110, 2053–2063
25. Jantzen, H. M., Chow, A. M., King, D. S., and Tjian, R. (1992) Genes Dev. 6, 1950–1963
26. Drakas, R., Tu, X., and Baserga, R. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 9272–9276
27. Hannan, K. M., Brandenburger, S. C., Jenkins, A., Shirk, K., Cavanaugh, A., Rothblum, L., Moss, T., Poortinga, G., McArthur, G. A., Pearl, R. B., and Hannan, R. D. (2003) Mol. Cell. Biol. 23, 8862–8877
28. Tuan, J. C., Zhai, W., and Comai, L. (1999) Mol. Cell. Biol. 19, 2872–2879
29. Voit, R., Kuhn, A., Sander, E. E., and Grummt, I. (1995) Nucleic Acids Res. 23, 2593–2599