Identification and Characterization of Human Cdc7 Nuclear Retention and Export Sequences in the Context of Chromatin Binding

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The Cdc7 serine/threonine kinase activates the initiation of DNA replication by phosphorylating MCM proteins that are bound to the origins of DNA replication. We reported previously that human Cdc7 nuclear import is mediated directly by importin-β through its binding to the Cdc7 nuclear localization sequence (NLS). Here, we report that human Cdc7 nuclear localization is regulated by two additional elements: nuclear retention (NRS) and export sequences (NES). Cdc7 proteins imported into the nucleus are retained in the nucleus by associating with chromatin, for which NRS-(306–326) is essential. Importantly, this binding appears to be specific to the origin of DNA replication, because the binding of wild-type Cdc7 to origin is 2.4-fold higher than to non-origin DNA. Furthermore, an NRS-defective Cdc7 mutant could not be retained in the nucleus, although it was imported into the nucleus normally. Together, our data suggest that NRS plays an important role in the activation of DNA replication by Cdc7. The Cdc7 proteins unassociated with chromatin are bound by CRM1 via two NES elements: NES1 at 458–467 within kinase insert III, and NES2 at 545–554 within the kinase IX domain. The primary function of the Cdc7-CRM1 association may be to translocate nuclear Cdc7 to the cytoplasm. However, the binding of CRM1 with Cdc7 at NES2 raises an interesting possibility that CRM1 may also down-regulate Cdc7 by masking its kinase domain.

The entire genome in the eukaryotic cell is replicated only once per cell cycle, mainly through the regulation of the pre-replication complex (pre-RC)3 (1–3). A pre-RC is sequentially formed at the origin of DNA replication (ori) during late M and early G1 phase. Two key proteins in this process are Cdc6 and Cdt1, of which availability is the limiting step for loading of the licensing MCM factors (2, 4, 5). Once the MCM protein complex is loaded onto an ori, Cdc6 and Cdt1 may be dissociated from the ori and inactivated by degradation or by association with other proteins to prevent re-replication (2, 5–7). Although the formation of pre-RC and the function of cyclin-dependent kinases are essential, these are not sufficient for the activation of replication initiation. Cdc7 Dbf4 serine/threonine kinase has to phosphorylate MCM proteins bound on the chromatin at oris to finalize the replication activation process (2, 8–16). Therefore, the catalytic Cdc7 and the regulatory Dbf4 subunits must enter the nucleus and bind to chromatin at an ori prior to the activation of replication initiation.

Human Cdc7 (huCdc7) protein comprises 11 kinase domains and two kinase inserts (17, 18). The kinase domains are highly conserved in all species from yeast to human, while the amino acid sequences of Kinase Insert II (amino acids, 275–368) and III (440–538) are more diverse (existence of Kinase Insert I is presently unknown).

Masai and co-workers (12, 20) demonstrated that huCdc7 associates with chromatin immediately after reformation of the nuclear membrane in early G1, while association of Dbf4 with chromatin occurs in late G1. This observation suggests that the association of Cdc7 with chromatin is independent of Dbf4 in human cells. However, the mechanism how Cdc7 binds to chromatin to activate DNA replication in mammalian cells is presently unknown.

Here, we report that huCdc7 nuclear localization is regulated by nuclear import, chromatin binding, and nuclear export. These three regulation steps are mediated by nuclear localization sequence (NLS), nuclear retention sequence (NRS), and nuclear export sequence (NES), respectively. The nuclear retention sequence (amino acids 306–326) is present within the origin of DNA replication (ori) during late M and early G1 phase. Two key proteins in this process are Cdc6 and Cdt1, of which availability is the limiting step for loading of the licensing MCM factors (2, 4, 5). Once the MCM protein complex is loaded onto an ori, Cdc6 and Cdt1 may be dissociated from the ori and inactivated by degradation or by association with other proteins to prevent re-replication (2, 5–7). Although the formation of pre-RC and the function of cyclin-dependent kinases are essential, these are not sufficient for the activation of replication initiation. Cdc7 Dbf4 serine/threonine kinase has to phosphorylate MCM proteins bound on the chromatin at oris to finalize the replication activation process (2, 8–16). Therefore, the catalytic Cdc7 and the regulatory Dbf4 subunits must enter the nucleus and bind to chromatin at an ori prior to the activation of replication initiation.

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* This work was supported by grants from the Canadian Institutes of Health Research (MOP79473) and the Natural Sciences and Engineering Research Council of Canada (203528-02) (to H. L.). This report is a part of the PhD thesis of B. J. K. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

1 Supported in part by an Ontario Graduate Scholarship and the University of Ottawa Excellence Award.

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3 The abbreviations used are: pre-RC, pre-replication complex; CE, cytoplasmic extracts; ChIP, chromatin immunoprecipitation; LMB, Leptomycin B; MCM, minichromosome maintenance; NES, nuclear export sequence; NLS, nuclear localization sequence; NRS, nuclear retention sequence; ori, origin of DNA replication; WNE, whole nuclei; WT, wild type; GST, glutathione S-transferase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein.
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the previously identified NLS-(203–370). We show here for the first time that huCdc7 binds to chromatin at an ori, for which the NRS is essential. The huCdc7 NES comprises two leucine-rich motifs within the C-terminal region. Although each NES motif can mediate nuclear export at low efficiency, both NES1 and 2 elements are required for an effective Cdc7 nuclear export to the cytoplasm.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa and HEK 293T cells were maintained in Dulbecco’s-modified Eagle’s Medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and gentamycin (50 μg/ml) (Invitrogen, Burlington, Ontario, Canada). Chinese hamster ovary (CHO) cells were grown in Minimal Essential Medium supplemented with 10% Fetal Clone II (HyClone). Cells grown on a glass coverslip were transfected with plasmids using Lipofectamine PLUS™as suggested by the supplier (Invitrogen).

Plasmid Constructs—The pEGFP-huCdc7 recombinant plasmid was constructed by cloning a full-length cDNA encoding the entire human Cdc7 into the Smal site of pEGFP-C1 (Clontech) (19). Various Cdc7 deletion mutants were generated by PCR-based cloning using pfu DNA polymerase (Invitrogen). Alternatively, DNA fragments generated by digestion with restriction endonuclease(s) were cloned into pEGFP-C1 (Clontech). The substitutions of amino acid sequence from a leucine to an alanine within Cdc7 NESs were generated by site-directed mutagenesis. The following oligonucleotides (all from IDT, Coralville, IA) were used for mutagenesis: sense 5′-GATAAAGCTTCTAGATCTAAATCCA-3′ and antisense 5′-TCGACTTCAGTTCAAGTTTCTTCAAGGAG-3′. To generate tandem GFP-tagged (2×GFP) constructs, pEGFP-Cdc7NES1, pEGFP-Cdc7NES2, pEGFP-Cdc7NES1-NES2, and pEGFP-MEKKNES were digested with AgeI and then ligated with a 790-bp fragment generated by double-digesting pEGFP-C1 with AgeI and XmaI. pGEX-huCdc7 recombinant plasmid was constructed as described previously (19). Various Cdc7 deletion mutants were generated by cloning mutant DNA fragments into pGEX-2T or pGEX-5X-1 (Amersham Biosciences) and described previously (19). GST pull-down assays were carried out using beads conjugated with glutathione-Sepharose 4B as suggested by the manufacturer (Amersham Biosciences) and described previously (19). GST pull-down assays were carried out using beads conjugated with either GST (negative control) or GST-importin-β recombinant proteins. Cells transfected with plasmids were lysed at 24 h post-transfection with Lysis buffer containing 150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-Cl (pH 7.5), 50 mM NaF, 50 mM glycerophosphate, 2 mM EDTA, 10% glycerol plus protease inhibitor mixture (Roche Applied Science). The cell lysates were then incubated for 4 h at 4 °C with glutathione-Sepharose 4B beads conjugated with GST or GST-importin-β.

Indirect Immunofluorescence Analysis—Cells grown on a glass coverslip were fixed with 4% paraformaldehyde for 10 min at room temperature, and were permeabilized with 0.1% Triton X-100 in PBS for 3 min. After treatment with PBS containing 1% bovine serum albumin for 2 h at room temperature, cells were incubated with primary antibodies for 90 min, followed by three washes with PBS (5 min for each wash) and incubation with fluorescence-conjugated secondary antibodies for 45 min at room temperature. Subsequently, cells were washed with PBS, mounted on a glass slide, and then visualized by fluorescence microscopy (Axiovert 100, Carl Zeiss). Hoechst 33258 (Sigma) was used to visualize the nuclei.

Chromatin Association Assays—Chromatin fraction described in Fig. 3 was prepared as described previously with some modifications (23–25). Briefly, cells were lysed in the Nuclei Isolation buffer (NIB; 15 mM Tris-HCl pH 7.4, 60 mM KCl, 15
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Briefly, HeLa cells grown on a glass coverslip were washed with PBS and extracted for 2 min at room temperature with high salt buffer containing 50 mM HEPES (pH 6.9), 10 mM EGTA, 2 mM MgCl₂, 350 mM NaCl, 0.5% Triton X-100 and protease inhibitor mixture. After washing with PBS, cells were fixed (i.e. X100/PFA), paraformaldehyde washed off, blocked with 1% bovine serum albumin in PBS, and incubated with purified recombinant GST-Cdc7 WT or GST-Cdc7 mutant proteins for 2 h at room temperature. After washing with PBS, cells were incubated with FITC-conjugated anti-GST antibodies for 50 min at room temperature, followed by three washes with PBS. When cells were fixed prior to the treatment of the cells with high salt extraction buffer containing Triton X-100 (see above) is defined as PFA/X100.

**Chromatin Immunoprecipitation (ChIP) Assay**—Cells transfected with expression plasmids for 12 h were cross-linked for 10 min with 1% formaldehyde, followed by three washes with cold PBS. Cells were then scraped off, resuspended in Lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% SDS, and protease inhibitor mixture), and incubated on ice for 15 min. Subsequently, chromatin was fragmented by sonication on ice using a Sonic Dismembrator (Fisher Scientific), each for 10 s 8 times at 15% amplification. Soluble fractions were collected by centrifugation at 13,000 rpm in a benchtop centrifuge for 5 min at 4 °C, and were then diluted 10-fold with Dilution buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 0.5% SDS, and protease inhibitor mixture). The diluted fractions were incubated with agarose conjugated with anti-GFP antibody to immunoprecipitate GFP-tagged wild type (WT) and mutant Cdc7 proteins. The beads were washed twice with low-salt buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and 150 mM NaCl), twice with high salt buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% Triton X-100, 1% SDS, and 500 mM NaCl), twice with LiCl buffer (10 mM Tris-HCl, pH 8.0, 1.25 mM LiCl, 1% Nonidet-40, 1% sodium deoxycholate, and 1 mM EDTA), and three times with TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). All buffer used was supplemented with protease inhibitor mixture. Approximately 10% of total beads were used for Western blot analysis, and the rest was incubated with Elution buffer (1.0% SDS and 0.1 mM NaHCO₃). The NaCl concentration of the eluted sample (i.e. protein-DNA complexes) was adjusted

![FIGURE 1. Human Cdc7 amino acid sequence spanning 300–332 is required for its nuclear retention. A, schematic presentation of Cdc7 deletion mutants used for identification of amino acid sequence required for its nuclear localization. N and C indicate nuclear and cytoplasmic localization, respectively. B, subcellular localizations of WT and mutant GFP-Cdc7 proteins in CHO cells transfected with the construct indicated in each panel. C, Cdc7(Δ300–312) efficiently binds to importin-β. Purified GST-importin-β proteins were immobilized on glutathione-Sepharose 4B beads. The beads were then incubated with total CHO cell lysates containing GFP (lanes 1 and 4), GFP-Cdc7 (lanes 2 and 5), or GFP-Cdc7(Δ300–332) (lanes 3 and 6) proteins. Proteins bound to the beads were detected by Western blotting with an anti-GFP antibody. Coomassie, Coomassie Blue-stained gel (low panel). D, GFP-Cdc7(Δ300–332) protein was localized in the nucleus in the presence of 25 µM LMB (lanes 3 and 6) or LMB (lanes 5 and 8) at 2-h post-transfection. E, subcellular distributions of Cdc7(Δ300–332) in cell populations treated with or without LMB (at least 100 cells were examined). N + C, diffusely localized throughout the cell.](image-url)
to 0.2 M, followed by incubation at 65 °C overnight. The sample was then further incubated with proteinase K (100 μg/ml) for 2 h at 45 °C. DNA was purified by phenol chloroform extraction and resuspended in DNase-free water, which was subjected to quantification by real-time PCR (7900HT, Applied Biosystems) using SYBR green master mix (Applied Biosystems). Standard curves were obtained from a serial dilution of HEK 293T genomic DNA (0.2, 1, 5, 10, and 50 ng) as template. The following primers at the origin of the MCM4 gene locus were used as described previously (27). UPR-F: 5′−AAACCAGAAGT-AGGCTCTGCTGG-3′ and UPR-R: 5′−GGCCAGTTAAAGCGGCTCTTTGGG-3′. An exon 9 primer set was used as a negative control (EX9-F: 5′−ATGCTTCCGGAGACTCTGAAGC-3′ and EX9-R: 5′−GGCCTCTATTCTGAGATCATGC-3′) (27). In the case of endogenous huCdc7, fragmented chromatin was incubated with an anti-Cdc7 antibody at 4 °C overnight. Subsequently, chromatin-Cdc7 antibody complex was incubated with protein A- and G-agarose/salmon sperm DNA (Upstate) for 2 h. As a negative control, protein A- and G-agarose/salmon sperm DNA and mouse IgG were used. The beads were then washed as described above.

**Co-immunoprecipitation Assays**—Cells were transfected with expression plasmids for 12 h, washed three times with PBS, resuspended with cold Lysis buffer (50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA, 10% glycerol, and protease inhibitor mixture), and then incubated at 4 °C for 30 min with rotation. The supernatant was separated by centrifugation at 12,000 rpm in a bench-top centrifuge for 10 min at 4 °C, and then incubated with cold Lysis buffer and resuspended in the SDS sample buffer, followed by SDS-PAGE Western blot analysis.

**RESULTS**

**Identification and Characterization of the Cdc7 NRS**—We previously reported that the human Cdc7 Kinase Insert II domain (amino acids, 203–370) is required for its binding to importin-β, by which Cdc7 is imported into the nucleus. To further dissect Cdc7 NLS, we generated and characterized several mutant constructs bearing a series of deletions within the Kinase Insert II domain (Figs. 1A and supplemental S1A). We found that the mutant bearing a deletion from 300 to
332 (i.e. Cdc7(Δ300–332)) was localized in the cytoplasm (Figs. 1, A and B, and supplemental S2), suggesting this segment is critical for Cdc7 nuclear localization. To further specify a segment critical for nuclear localization, we generated and characterized several mutants bearing smaller deletions within the 300–332 segment. The Cdc7(Δ301–313) and Cdc7(Δ319–326) mutants were localized in the nucleus, while Cdc7(Δ301–326) was mainly in the cytoplasm, suggesting amino acids 314–318 is absolutely required for Cdc7 nuclear localization (Fig. 1, A and B). However, Cdc7(Δ306–313) was diffusely localized throughout the entire cell (data not shown), suggesting that amino acids 314–318 is required but not sufficient for Cdc7 nuclear localization. The 306–326 segment may be the minimum sequence sufficient for Cdc7 nuclear retention since the Cdc7(Δ306–326) mutant was mostly localized in the cytoplasm (Fig. 1B). Nevertheless, considerable amount of Cdc7(Δ306–326) proteins was still detected in the nucleus. Therefore, we conclude that the 300–332 segment contains the intact Cdc7 NRS.

We found previously that the binding of Cdc7 to importin-β is essential for its nuclear import. Therefore, we examined the binding affinity of Cdc7(Δ300–332) for importin-β. To our surprise, the binding affinity of this mutant for importin-β was not notably different from that of GFP-Cdc7 WT (Fig. 1C). This data raised the possibility that GFP-Cdc7(Δ300–332) mutant was normally imported into the nucleus by importin-β. However, the mutant protein could be localized mainly in the cytoplasm due to the lack of nuclear retention and subsequent nuclear export. To test this hypothesis, we analyzed the nuclear localizations of Cdc7 WT and Cdc7(Δ300–332) mutant proteins after cells were treated with Leptomycin B (LMB), an inhibitor of protein nuclear export by CRM1/exportin 1 (28, 29). As shown in Fig. 1D, GFP-Cdc7(Δ300–332) protein was localized in the nucleus in the presence of LMB. These data, in conjunction with Fig. 1C (i.e. effective binding of Cdc7(Δ300–332) with importin-β), strongly suggest that the cytoplasmic localization of Cdc7(Δ300–332) is not due to defects in nuclear import but due to the combination of deficient nuclear retention and effective nuclear export.

To gain a better understanding of Cdc7 nuclear retention, we examined the subcellular distribution patterns of the GFP-Cdc7(Δ300–332) mutant in a cohort of a cell population. As shown in Fig. 1E, GFP-Cdc7(Δ300–332) was exclusively localized in the nuclei of more than 60% of cells treated with LMB, compared with only 2% of the LMB-nontreated control. Interestingly, the same survey revealed that GFP-Cdc7(Δ300–332) was localized in the nuclei as well as in the cytoplasm of ~40% cells with or without LMB treatments. Although the exact reason for this is presently unknown, it is possible that the nuclear import and export of GFP-Cdc7(Δ300–332) proteins are in equilibrium in a certain cell population (at a given time). Alternatively (or in combination), LMB might not be able to block the nuclear export of all the Cdc7(Δ300–332) proteins under certain conditions. Furthermore, part of the signals detected in the cytoplasm might be due to the presence of GFP-Cdc7(Δ300–332) cleavage products as shown in Fig. 1C (lane 3) (note that anti-GFP antibodies were used for the work shown in Fig. 1, C, lanes 1–3 and E).

**Cdc7 Nuclear Retention Is Mediated by Associating with Chromatin via Its NRS**—We wanted to examine the subcellular localization of Cdc7(Δ300–332) mutant by an alternative approach. We therefore established a novel in vitro experimental system by removing all the cellular soluble proteins, and the plasma membrane while leaving the chromatin and nuclear matrix integrity largely intact. This was achieved by treating cells with Triton X-100 and removing the soluble cellular fraction as described under “Experimental Procedures.” If these “ghost nuclei” are incubated with proteins of interest, the proteins would freely diffuse in and out of the nucleus without the involvement of cells’ import and export machineries. Subsequently, all the proteins, except those associated with chromatin and nuclear matrix, can be washed off. We examined the validity of this experimental system by determining the subcellular localization of importin-β (a known cytoplasmic protein) and MCM2 (a known chromatin-associating protein) (16, 30, 31). As expected, the endogenous importin-β was effectively washed off when cells were treated with Triton X-100 prior to fixation (Fig. 2A, X100/PFA). In contrast, the endogenous MCM2 proteins were not released from the cell under the same conditions due to their association with chromatin (Fig. 2B).
We also found that Orc2, a replication protein binding to chromatin, showed a similar chromatin association pattern under the same conditions (data not shown). Therefore, we concluded that this in vitro assay system is a reliable method for studying protein-chromatin/nuclear matrix association. Using this assay system, we examined the nuclear retention of wild type Cdc7,
mutant proteins were detected only in the CE fraction in the absence of LMB. Even in the presence of LMB, GFP-Cdc7(Δ300–332) was largely absent from the Chr fraction. Our data shown in Figs. 1, 2, 3, and supplemental S3 collectively suggest that the NRS element (300–326) is required for Cdc7 binding to chromatin.

Cdc7 Binds to Chromatin Preferentially at an ori through Its NRS—Although Cdc7 is thought to associate with chromatin at or near oris to initiate DNA replication (15, 16, 33, 34), this is yet to be demonstrated in mammalian cells. Therefore, we carried out chromatin immunoprecipitation (ChIP) assays to examine whether mammalian Cdc7 binds preferentially to a known ori. Under our ChIP assay conditions, the anti-Cdc7 (Fig. 4B) and -GFP antibodies (Fig. 4D) could effectively immunoprecipitate Cdc7 and GFP/GFP fusion proteins, respectively. Therefore, using these antibodies and the MCM4 replicon model (27), we determined the abundance of DNA bound to Cdc7 WT or Cdc7(Δ300–332) mutant as described under “Experimental Procedures.” As shown in Fig. 4C, endogenous Cdc7 associated with the MCM4 ori 2-fold more frequently than with the exon 9 non-ori region, suggesting that Cdc7 binds preferentially to ori over non-ori regions. To examine the role of NRS in the Cdc7 binding to an ori/chromatin, we then carried out a similar ChIP assay using HEK 293T cells transfected with either Cdc7 WT or the NRS-defective Cdc7(Δ300–332) construct. As expected, Cdc7 WT was associated with the MCM4 ori 2.4-fold more frequently than with the exon 9 region, while Cdc7(Δ300–332) mutant did not show any preference (Fig. 4E). These data, therefore, suggest that Cdc7 binds to chromatin preferentially at an ori, and NRS is required for this preferential binding.

Human Cdc7 Contains Two Nuclear Export Signals That Are Recognized by CRM1/Exportin 1—The data shown in Figs. 1–3 suggest the existence of active nuclear export mechanism(s) for Cdc7 protein, perhaps by interactions of Cdc7 with CRM1 through a specific sequence. To test this hypothesis, we generated several GFP-tagged Cdc7 deletion mutants and examined their nuclear localization in the presence or absence of LMB (Fig. 5, A and B). GFP-Cdc7-(432–574) localized in the cytoplasm while GFP-Cdc7-(1–431) was in the nucleus, suggesting that the segment spanning amino acids 432–574 contains nuclear export element(s) (Fig. 5, A and B). We found that both GFP-Cdc7-(511–574) and GFP-Cdc7-(432–510) proteins localized mainly in the cytoplasm in the absence of LMB. However, these proteins were localized mostly in the nucleus in the presence of LMB (Fig. 5, A and B). (It should be noted that these proteins are small enough to diffuse in and out of the nucleus quite freely.) By contrast, GFP-Cdc7-(563–574) diffusely localized throughout the entire cell in the presence or absence of LMB, suggesting that it contains neither NLS nor NES. Together, these data suggest that two separate NESs may be necessary for the nuclear export of Cdc7 in mammalian cells.
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present within the segment spanning amino acids 432–563: one NES may be within the amino acids 432–510 segment and the other within 511–574 (Fig. 5, A and B). Because the subcellular localization of the Cdc7(Δ300 – 332) NRS-defective mutant is dependent upon the presence of LMB, Cdc7 nuclear export may be mediated by CRM1/exportin 1.

To gain insight into the precise locations of two NESs, we compared human and other mammalian Cdc7 sequences with known NESs (Fig. 5C). The Cdc7 amino acid sequences 458–467 and 545–554 showed high similarity with the known nuclear-export consensus sequence (Fig. 5C). Therefore, they were tentatively named NES1 and NES2, respectively. In addition, Leu465 (underlined in Fig. 5C) was predicted to be within an NES by NES-prediction software (35). Therefore, we generated and examined cellular localization of several constructs that contain 2× GFP plus amino acids 458–467, 545–554, or both (Fig. 5, D and E). An NES was previously reported to retain its nuclear export activity when it is tagged with 2× GFP (21, 36, 37). Consistent with these previous reports, the 2×GFP-MAPKKNES positive control was exclusively localized in the cytoplasm in the absence of LMB, compared with non-specific subcellular localization of GFP alone and GFP plus a scrambled peptide (Fig. 5, D and E). When cells were treated with LMB, however, the 2×GFP-MAPKKNES fusion proteins mostly localized in the nucleus. The 2×GFP-Cdc7(456–467, 545–554) fusion protein showed exactly the same subcellular localization pattern as 2×GFP-MAPKKNES in the absence or presence of LMB (Fig. 5, D and E). Unexpectedly, the subcellular localization pattern of either 2×GFP-NES1 or 2×GFP-NES2 alone remained unchanged in the absence or presence of LMB (Fig. 5, D and E). Taken together, these data suggest that both the NES1 and 2 are required for efficient nuclear export, although NES1 and 2 each contains a weak separate NES element (Fig. 5C).

To further characterize Cdc7 NESs, we examined the subcellular localization of several Cdc7(Δ300–332) mutant proteins containing additional point mutations within the NES1 and/or NES2 (Fig. 6A). We used Cdc7(Δ300–332) as the base of NES mutant constructs to avoid potential complication by the presence of NRS. As shown in Fig. 6, A and B, the Cdc7(Δ300–332, L465A/L549A/L550A) mutant was mainly localized in the nucleus, while the Cdc7(Δ300–332, L459A/L550A), and Cdc7(Δ300–332, L465A) mutants were localized in the cytoplasm or diffusely throughout the cell, respectively. (Note that Leu465 is located within NES1, and Leu459 and Leu550 within NES2). This result is consistent with data shown in Fig. 5, and confirms that both NES1 and 2 are required for effective nuclear export of the Cdc7 protein.

To examine whether Cdc7 binds to CRM1 in vivo, we carried out an immunoprecipitation assay (Fig. 6C). CRM1 was co-precipitated with GFP-Cdc7 or GFP-Cdc7(Δ300–332) (Fig. 6C, lanes 2 and 3). The Cdc7(Δ300–332, L465A/L549A/L550A) mutant also bound to CRM1, but with significantly reduced affinity (~30% of the Cdc7(Δ300–332)) (Fig. 6D). This result is consistent with the data observed by microscopy of live cells, which showed a portion of Cdc7(Δ300–332, L465A/L549A/L550A) mutant is also localized in the cytoplasm (Fig. 6B).

DISCUSSION

The Regulation of Cdc7 Nuclear Import and Export—We previously reported that human Cdc7 is imported into the nucleus by importin-β, for which the NLS at the Cdc7 Kinase Insert II domain (amino acids 203–370) is essential (19). Because we did not know at the time that an NRS was localized within the Cdc7 NLS, we thought that Cdc7(Δ300–332) was localized in the cytoplasm due to low affinity of this mutant for importin-β. However, the binding affinity of Cdc7(Δ300–332) for importin-β was not notably diminished (Fig. 1C). Therefore, we thought that the cytoplasmic localization of Cdc7(Δ300–332) was not due to defects in nuclear import but due to other mechanisms such as the nuclear retention signal. This hypothesis turned out to be correct because Cdc7(Δ300–332) mutant was predominantly localized in the nucleus in the presence of LMB (Fig. 1D). We therefore conclude that Cdc7(Δ300–332) mutant contains an intact nuclear import signal; however, it lacks a functional nuclear retention signal.

In our previous report (19), we reported that Lys306 and Lys309 within the NLS were critical for nuclear localization, since the K306A/K309A double point mutant was localized in the cytoplasm and showed low affinity for importin-β. This data appears to be contradictory with our current observation that Cdc7(Δ300–332) has high affinity for importin-β as Cdc7 WT (Fig. 1C). Therefore, we compared side-by-side the
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affinity of Cdc7(Δ300–332) and Cdc7K306A/K309A for importin-β. Consistent with our previous data, Cdc7(Δ300–332) showed ~10-fold higher binding affinity for importin-β than Cdc7K306A/K309A (supplemental Fig. S4). Presently, we cannot explain these seemingly contradictory data with respect to their different binding affinities for importin-β. However, it is possible that certain amino acids within the 300–332 segment may counteract with other part of NLS and negatively function in the formation of a complex with importin-β (and thus down-regulate Cdc7 nuclear import). This hypothesis is supported by the published data that introduction of additional mutations could rescue dimerization-defective mutants of RelB and p50 (38).

Histologically, the term NLS has been coined with amino acid sequences required for nuclear import (reviewed in Ref. 39). However, our data now suggest that an NLS may contain at least two different components since proteins imported into the nucleus have to be retained as long as they are needed to be in the nucleus (summarized in Fig. 7). We envision that nuclear import and retention could be mediated by a single NLS element or two physically separated elements. It should also be noted that identification/interpretation of an NLS can be missed if nuclear retention signal is defective.

Cdc7 Nuclear Export—Because we and others (17, 18, 20, 40) have shown previously that mammalian Cdc7 proteins localize predominantly in the nucleus, it was surprising to find that hUCdc7 contains two functional NESs. Although each NES shows weak nuclear export function, both NES1 (amino acids, 458–467) and NES2 (545–554) are required for the full nuclear export activity. NES1 is localized within Kinase Insert III, which is expected because regulatory elements are likely presented within a non-kinase domain. However, it was surprising to find that NES2 was localized within a kinase domain (i.e. Kinase domain IX). This data raises an interesting possibility that CRM1 can also down-regulate Cdc7 by masking its kinase domain.

Examination of the Cdc7 potential tertiary structure using the Network Protein Sequence Analysis program (41) suggests that each of the NES1 and 2 sequences may be positioned within a 12–13 amino acid-long α-helical stretch. Interestingly, the computer program also predicts that there is only one long-stretch of random coils between these two α-helices. This may indicate that NES1 and 2 could position closely on the tertiary structure, and thus the two sites together may form a binding site for CRM1. However, an accurate analysis of interactions between NESs and CRM1 cannot be determined at this point because crystallization of human Cdc7 has not yet been reported.

Cdc7 NRS and Activation of Replication—We have shown here for the first time that Cdc7 is associated with chromatin preferentially at an ori in mammalian cells (Fig. 4). This observation is consistent with the currently accepted model that Cdc7 kinase activates the initiation of DNA replication by phosphorylating MCM proteins bound at or near an ori. Furthermore, data obtained from our ChIP assays suggests that NRS is required for the association of Cdc7 with chromatin at an ori (Fig. 4E). The level of binding affinity between the NRS-defective Cdc7(Δ300–332) with an ori was equivalent with that between Cdc7 WT and the MCM4 exon 9 (i.e. non-ori), suggesting that the NRS-mediated binding of Cdc7 to an ori may be a specific interaction. Together, our data are consistent with the notion that NRS is required for the activation of DNA replication by Cdc7 kinase.

Acknowledgments—we thank Helen Zhao for generating several Cdc7 deletion mutants and Catharine Song for reading this manuscript.

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