Importin-β Mediates Cdc7 Nuclear Import by Binding to the Kinase Insert II Domain, Which Can Be Antagonized by Importin-α

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We investigated the nuclear import mechanism of Cdc7, which is essential for the initiation of DNA replication. Here we report that importin-β binds directly to Cdc7 via the Kinase Insert II domain, promoting its nuclear import. Although both importin-α and -β bind to Cdc7 via the Kinase Insert II domain in a mutually independent manner, the binding affinity of Cdc7 for importin-β is ~10 times higher than for importin-α at low protein concentrations of an equimolar ratio. Immunodepletion of importin-β, but not importin-α, abrogates Cdc7 nuclear import, and the addition of importin-β to the importin-depleted cytosol restores Cdc7 nuclear import. Furthermore, transduction of anti-importin-β, but not anti-importin-α antibodies, into live cells inhibits Cdc7 nuclear import. Unexpectedly, we found that Cdc7 nuclear import is inhibited by competitive binding of importin-α to Cdc7. Further studies by site-directed mutagenesis suggest that Lys\textsuperscript{306} and Lys\textsuperscript{309} within the Kinase Insert II domain are critical for Cdc7 nuclear localization.

The Cdc7-Dbf4 kinase functions as a molecular switch for the activation of individual origins of DNA replication (oriS) throughout S phase (1–6). To be functional, both the catalytic Cdc7 and regulatory Dbf4 subunits have to move into the nucleus, bind to chromatin, and associate with each other (7–9). Although Dbf4 contains a classical nuclear localization sequence (cNLS)\textsuperscript{2} (10), Cdc7 does not have any known NLS. It was initially suggested that Dbf4 binds and transports Cdc7 into the nucleus (11). However, more recent data have shown that Cdc7 is located in the nucleus even when Dbf4 is not produced (12–16).

The Cdc7 protein consists of 11 putative kinase domains that are highly conserved in all known Cdc7-related mammalian proteins (12, 15, 16). In addition, there are two kinase insert: the Kinase Insert II spans from amino acids 203 to 370, and the Kinase Insert III from 440 to 538 (12, 15–17). A potential short Kinase Insert I also exists at the amino acid residues 75–88. The amino acid sequences of the kinase inserts are the most diverse regions of the entire Cdc7 protein, and thus the inserts are thought to be involved in species-specific regulation and/or interactions with other proteins such as Dbf4 (4).

Proteins containing a cNLS are imported into the nucleus by an importin-α/β heterodimer (18–20). In this transportation mode, the cNLS in a cargo protein is recognized and bound by importin-α through its carboxy-terminal region, which, in turn, is bound by importin-β via its amino-terminal importin-β binding (IBB) domain. The cargocarrier-α/β complex is transported into the nucleus through the nuclear pore complex (NPC) (18, 20). Several recent data have demonstrated that certain proteins are transported into the nucleus by importin-α alone (21), or by importin-β alone (22–27).

We examined the regulatory mechanism of human Cdc7 (huCdc7) nuclear transportation using \textit{in vitro} and \textit{in vivo} assays. We found that huCdc7 is directly bound and translocated into the nucleus by importin-β. The binding site is mapped to the Cdc7 Kinase Insert II, and the Lys\textsuperscript{306} and Lys\textsuperscript{309} residues within this domain are critical for Cdc7 nuclear localization. Most interestingly, importin-α can competitively bind to the Cdc7 Kinase Insert II, and can thus effectively inhibit importin-β-mediated Cdc7 nuclear transportation. Our data also raises the possibility that the binding of Cdc7 to importin-α could be involved in the activation or maintenance of the replication checkpoint in response to cell damaging agents such as irradiation and anticancer agents.

**EXPERIMENTAL PROCEDURES**

Cell Culture and DNA Transfection—HeLa and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and a combination of penicillin (50 units/ml) and streptomycin (50 μg/ml). Chinese hamster ovary (CHO) cells were grown in minimal essential medium supplemented with 10% Fetal Clone II (HyClone). Cells grown on a coverglass were transfected with plasmids for 12 h using Lipofectamine PLUS™ reagent as suggested by the supplier (Invitrogen) and as described previously (28).

**Plasmid Constructs**—The pEGFP-huCdc7 and pCMV-huCdc7 recombinant plasmids were constructed by cloning a full-length cDNA encoding the entire human Cdc7 into the SmaI site of pEGFP-C1 (Clontech) or into the SmaI-XhoI site of pCMV-Tag2 (Stratagene), respectively. For recombinant protein extraction in bacteria, a full-length Cdc7 CDNA was cloned into the SmaI site of pGEX-2T. Various Cdc7 deletion mutants were generated by cloning mutant DNA fragments into pGEX-2T or pGEX-5X-1 vectors (Amersham Biosciences). The plasmids PEVRF-NLS-PCNA and pEGFP-C1-PCNA were a kind gift of Dr. M. C. Cardoso (Max-Delbrück-Center for Molecular Medicine) (29). The plasmid pGEX-KG-importin-β was a kind gift of Dr. S. A. Kornbluth (Duke University) (22). The plasmids...
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pQE70-importin-α2 (Rch-1/hSRPα1) and pQE60-importin-β were kindly provided by Dr. D. Görlich (University of Heidelberg) (30, 31). pGEX-2T-importin-α2 was constructed by subcloning the DNA segment spanning from BamHI to EcoRI of pQE70-importin-α2 into a pGEX-2T vector. pQE70-importin-α2 (AIBB; Δ amino acids 1–51) was constructed by subcloning a PCR fragment of pQE70-importin-α2 spanning SphI-BgIII into a pQE70 vector.

Antibodies—Ant-importin-β (C-19), -α1 (C-20), and -α2 (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or the BD Transduction Laboratories (610559 and R43020, respectively); antibodies against MCM2 (N-19), proliferating cell nuclear antigen (PCNA) (PC10), 14-3-3 (K-19), GFP (B-2), GST (Z-5), GST-fluorescein isothiocyanate (FITC) (Z-5), and all secondary antibodies were purchased from Santa Cruz. Anti-His tag polyclonal antibodies were purchased from Cell Signaling, and anti-Cdc7 antibodies (K0070–3) were purchased from MBL International (Woburn, MA).

Protein Expression and Purification—Recombinant GST, GST-Cdc7, GST-importin-α, and GST-importin-α2 proteins were expressed in Escherichia coli (BL21), and were purified by affinity chromatography on glutathione-Sepharose 4B as suggested by the manufacturer (Amersham Biosciences). GST pull-down assays were carried out using beads conjugated with either GST (negative control) or GST-Cdc7 recombinant proteins. Recombinant His-tagged proteins were purified using BD Talon metal affinity resins according to the manufacturers instruction (BD Biosciences) and as described previously (30, 31). Prior to carrying out nuclear import assays, all purified proteins were extensively dialyzed against Nuclear Transport Buffer (NTB: 20 mM HEPES (pH 7.3), 110 mM NaCl, 1 mM KCl, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and protease inhibitor mixture). GST Pull-down Analysis—Cells transfected with plasmid 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 glyceralphosphate, 2 mM EDTA, 10% glycerol plus 1× protease inhibitor mixture (Roche) (25). The cell lysates were then incubated for 4 h at 4°C with glutathione-Sepharose 4B beads pretreated with GST, GST-importin-α2, or GST-importin-β, respectively.

To identify the cellular proteins interacting with Cdc7, HeLa cells (106–108) were lysed with buffer containing 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Protein concentrations of cell lysates were determined with a Bio-Rad protein assay using a CONVERTIBLE filtration manifold system (Invitrogen), and concentrations of cell lysates were determined with a Bio-Rad protein assay. The beads were extensively washed with PBST (PBS containing 0.2% Triton X-100, 0.1 M NaCl, 1% Nonidet P-40, 0.5% SDS, 10% glycerol, and 0.5% β-mercaptoethanol).

Protein-Protein Interactions in Solution (“Solution Binding Assay”)—Purified recombinant His-importin-α2 or His-importin-β proteins were incubated for 30 min at room temperature with the glutathione-Sepharose 4B beads as above. The beads were extensively washed with PBST (PBS plus 0.02% Triton-X100), and the proteins bound to the beads were eluted by Elution Buffer (0.1 M glycine, 0.125 M NaCl, pH 2.8), to which 1.0 mM Tris buffer was added to adjust the pH to 7.0. The proteins eluted were concentrated using a Microcon YM-10 centrifugal filter unit (cut-off 10 kDa, Millipore), followed by resolving it with 2× SDS Sample Buffer containing 60 mM Tris-Cl (pH 6.8), 1% SDS, 10% glycerol, and 0.5% β-mercaptoethanol.

Dot-blot Analysis—Purified recombinant proteins were dot blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences) using a CONVERTIBLE™ filtration manifold system (Invitrogen), and the membrane was then air-dried for 40 min at room temperature. The membrane was “blocked” for 2 h with PBS buffer containing 0.2% Tween 20 and 5% nonfat dry milk, followed by incubation with recombinant importin-α2 (1 mg/ml) or importin-β (1 mg/ml) proteins at 4 °C overnight. The membrane was washed three times with PBST for 10 min each, and then proteins bound to the membrane were detected by Western blot analysis. Densitometry was carried out using the Quanti-Scan version 3.0 (Biosoft, Cambridge, United Kingdom).

Immunofluorescence Analysis—Cells grown on a coverglass were fixed with 4% paraformaldehyde for 10 min at room temperature, and were then permeabilized with 0.1% Triton X-100 in PBS for 3 min. After being treated with PBS containing 1% bovine serum albumin for 2 h at room temperature, cells were incubated with primary antibodies for 2 h at room temperature. The cells were washed three times with PBS (5 min each wash), and then incubated with FITC- or rhodamine-conjugated rabbit anti-mouse IgG for 45 min at room temperature. Subsequently, cells were washed with PBS, mounted on a slide glass, and then visualized by fluorescence microscopy (Axiovert 100, Carl Zeiss). Hoechst 33258 (Sigma) was used to visualize the nuclei.

In Vitro Nuclear Import Assay—Nuclear import assays in digitonin-permeabilized cells were performed as described previously (32) with minor modifications. Briefly, cells grown on a coverglass were permeabilized with cold NTB containing 40 μg/ml digitonin (Roche) for 5 min. The cells were washed once with cold NTB, and excessive buffer was removed. Subsequently, the cells on a coverslip were treated with a small amount of nuclear transport mixture and incubated at room temperature for 30 min. A complete nuclear transport mixture contained HeLa cell cytosol (8 mg/ml), recombinant GST-Cdc7 proteins, and an energy generating system (1 mM ATP, 5 mM creatine phosphate, 20 units of creating phosphokinase/ml) and protease inhibitor mixture (32). Nuclear import was determined by immunofluorescence using the FITC-conjugated anti-GST antibodies (Santa Cruz).

To deplete importin-α2 and/or importin-β, HeLa cell cytosol was incubated with 10 μg of anti-importin-α2 and/or 10 μg of anti-importin-β antibodies at 4 °C for 4 h, which was further incubated for another 1 h with protein-agarose A/G (Santa Cruz). The antibody-importin complexes bound to agarose beads were then removed by centrifugation at 1,000 × g for 2 min. This procedure was repeated three times to completely remove importin-α2 and/or -β proteins. For reconstitution experiments, purified recombinant importin-α2 (0.125 mg/ml) and/or importin-β (0.125 mg/ml) were added to the cytosol depleted of the importins. For the docking assay, recombinant importin-β was used as a source of import factor instead of HeLa cell cytosol, as previously described (30, 33, 34).

Chariot-based Antibody Transduction—HeLa and HEK293 cells grown on a coverglass were transduced with anti-importin-β or -importin-α2 antibodies using a Chariot™ transfection kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instruction. Normal goat IgG was used as negative control. At 12 h post-transfection, endogenous Cdc7 was detected by indirect immunostaining with an anti-Cdc7 antibody.

RESULTS

Both Importin-α2 and -β Interact with HuCdc7—We wanted to confirm the nuclear localization of tagged Cdc7 proteins at the onset of this investigation because several tagged recombinant Cdc7 proteins were to be used for this work. As expected, GFP-Cdc7 protein was localized in the nucleus (Fig. 1A). To learn how huCdc7 is transported into the nucleus, we analyzed the proteins associated with Cdc7 by a “pull-down” assay using glutathione-Sepharose beads coupled with recombinant GST or GST-Cdc7 as described under “Experimental Procedures.” PAGE and subsequent Western blot analysis of the proteins bound to the beads showed that importin-α2, -β, and MCM2 proteins interacted with Cdc7, whereas PCNA, 14-3-3β and importin-α1 did not (Fig. 1B;
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interacts with either importin-α2 or -β independent of the other importin. The dot-blot analysis using purified proteins also showed a similar result (Fig. 2D). Because the data in Fig. 2C appears to suggest that Cdc7 has higher affinity for importin-β than for -α (compared lanes 3 and 4), we carried out a more detailed dot blot experiment by applying different amounts and molar ratios of purified recombinant His-importin-β and His-importin-α2 onto each dot on a membrane. The membrane was then incubated with GST-Cdc7, followed by Western blot analysis with an anti-GST antibody. As shown in Fig. 2, E and F, the binding affinity of Cdc7 for both importin-α2 and -β is dose-dependent. However, the affinity of Cdc7 for importin-β is significantly higher than for importin-α2 at low protein concentrations. For example, the relative signal intensity of Cdc7 bound to importin-β at the concentration of 0.05 µg/dot was 83 (arbitrary) units, whereas that of importin-α2 at the same concentration was 17 units (Fig. 2, E and F). Furthermore, 0.25 µg/dot of importin-α2 was required to reach the relative intensity of 75 units (Fig. 2F). Considering the fact that the same amount of importin-α2 protein contains twice as many molecules than importin-β (i.e. molar ratio is 2:1 at the same amount of importin-α2 and -β proteins), the binding affinity of Cdc7 for importin-β is ~10-fold higher than for importin-α2 at low protein concentrations (0.05–0.25 µg/blot). Taken together, the data shown in Figs. 1 and 2 suggest that Cdc7 has high binding affinity for importin-β in the absence or presence of a low concentration of importin-α2, raising the possibility that importin-β is responsible for Cdc7 nuclear import.

Importin-β, but Not Importin-α, Mediates Cdc7 Nuclear Import—To test the hypothesis that importin-β is directly responsible for Cdc7 nuclear import, we carried out a series of in vitro nuclear import assays in the presence or absence of importin-β as described below. As shown in Fig. 3A, HeLa cell cytosol can effectively transport Cdc7 in the digiton-permeabilized cells, confirming that the HeLa cell cytosol contains all the necessary factors for Cdc7 nuclear import. To identify cytosolic factors responsible for Cdc7 nuclear transportation, we carried out an in vitro nuclear import assay using HeLa cytosol depleted of both importin-α2 and -β. This depleted cytosol did not support Cdc7 nuclear import (Fig. 3B, panels I and II). As expected, Cdc7 nuclear localization was restored when both importin-α2 and -β recombinant proteins were added back to the depleted cytosol (Fig. 3B, panels III and IV). Supple-
importin-α2 or -β alone from the “complete” HeLa cytosol. Consistent with the data shown in Fig. 3B, the absence or presence of importin-α2 did not affect Cdc7 nuclear import (Fig. 3C, III–VI). In contrast, the cytosol lacking importin-β could not support Cdc7 nuclear transport, which was restored when purified recombinant importin-β was added (Fig. 3C, VII–X). This result further confirms that importin-β, but not importin-α2, is responsible for Cdc7 nuclear import, although both can bind to huCdc7. Note that the depletion of the proteins was confirmed by Western blot analysis (Fig. 3D).

To examine whether importin-β also mediates Cdc7 nuclear import in vivo, we transduced normal IgG, anti-importin-α2, or anti-importin-β antibodies into HeLa cells using a Chariot™ transduction system (Active Motif). The transduction of nonspecific IgG and anti-importin-α2 antibodies into HeLa cells did not affect the nuclear localization of endogenous Cdc7 (Fig. 4, I–VIII). In contrast, the transduction of anti-importin-β antibodies into HeLa cells effectively inhibited Cdc7 nuclear import (Fig. 4, IX–XII), confirming that importin-β is responsible for Cdc7 nuclear import in vivo. We also carried out a similar experiment using HEK293 cells, and found exactly the same result as shown in Fig. 4 (data not shown). Based on the data obtained from in vitro and in vivo assays, we concluded that importin-β, but not importin-α, directly mediates Cdc7 nuclear import. This conclusion is further supported by the data obtained by “docking” assay (below).

**FIGURE 4.** Anti-importin-β (imp) antibody, but not anti-importin-α2 antibody, inhibits Cdc7 nuclear import in vivo. HeLa cells (or HEK293, which is not shown) were transduced with an anti-importin-α2 or -importin-β antibodies, and the location of endogenous Cdc7 was determined with an anti-Cdc7 antibody (I, V, and IX). Transduced anti-importin-α2 (V) and -importin-β (IX) antibodies were detected by rhodamine-conjugated anti-goat antibodies. The merged images of Cdc7, rhodamine, and Hoechst (DNA) are shown in panels IV, VIII, and XII. Normal goat IgG was used as a control (I).

**FIGURE 5.** The Cdc7 Kinase Insert II domain is required for interaction of Cdc7 with importin-β and its nuclear localization. A, a schematic representation of wild-type huCdc7 and several deletion mutants used in this study. B, purified recombinant His-importin-β (1 μg) or His-importin-α2 (0.5 μg) proteins were incubated with glutathione-Sepharose beads coupled with wild-type GST-Cdc7 or mutant GST-Cdc7 proteins (10 μg). Subsequently, proteins bound to the beads were analyzed by PAGE-Western blot analysis using an anti-His antibody (upper panels). Total input wild-type GST-Cdc7 and mutant GST-Cdc7 detected by anti-GST antibodies are shown in the lower panels. C, the binding ability of Cdc7 for importin-β is directly correlated to the docking of Cdc7 to the NPC (arrows). Note that a blown-up image is present as supplemental Fig. 1 to show the difference between proteins in the cytoplasm and docked at the nuclear membrane. D, the GFP-huCdc7 K306A,K309A double mutant is localized in cytoplasm. The GFP-Cdc7 K306A,K309A or K306A,K309A double mutant was transfected into CHO cells and the subcellular locations of these Cdc7 mutants were analyzed at 15 h post-transfection by fluorescence microscopy. E, the Cdc7 K306A,K309A mutant has significantly lower binding affinity for importin-β than wild type. The experiment was carried out similarly as described in Fig. 2A. Imp-β, immobilized recombinant GST-importin-β; Total, total proteins extracts from CHO cells transfected with GFP-Cdc7 wild-type or GFP-Cdc7 K306A,K309A mutant; Bound, GFP-Cdc7 wild-type or GFP-Cdc7 K306A,K309A proteins bound to the immobilized recombinant GST-importin-β.

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The Kinase Insert II Is Required for the Binding of Cdc7 to Importin-β and Subsequent Nuclear Import—We next wanted to learn the mechanism of how importin-β mediates Cdc7 nuclear import. To this goal, we generated several Cdc7 deletion mutants (Fig. 5A; data not shown), and then examined their ability of binding to importin-β. As shown in Fig. 5B, GST-Cdc7(Δ1–146) has full binding affinity for both importin-α2 and -β, suggesting there is no binding site within the first 146 amino acids at the NH₂ terminus. In contrast, GST-Cdc7(Δ196–372) (i.e. Kinase Insert II deletion) and GST-Cdc7(Δ1–431) completely lost Cdc7 binding affinity for both importin-α2 and -β, suggesting that the region encompassing amino acid residues 196–372 (i.e. Kinase Insert II domain) is required for Cdc7 binding to both importin-α2 and -β. The GST-Cdc7(Δ279–574) or GST-Cdc7(Δ1–279) alone bound to importin-β, albeit at a lower level. However, neither GST-Cdc7(Δ279–574) nor GST-Cdc7(Δ1–279) could bind to importin-α2 (Fig. 5B).

To determine whether the in vitro binding of Cdc7 to importin-β is directly relevant to Cdc7 nuclear transportation, we carried out in vitro docking assays. Digitonin-permeabilized HeLa cells were incubated with GST-Cdc7 or GST-tagged Cdc7 deletion mutants in the presence of purified recombinant His-importin-β and an energy generating system (note that cytosol was not used in this experiment). Because Ran protein and GTP were not added, the cargo protein (Cdc7) was not expected to be in the nucleus but “docked” at the NPC by this assay system (18, 20). Consistent with in vitro binding experiments, wild-type Cdc7 and Cdc7(Δ1–146) efficiently docked at the NPC in the presence of importin-β, but Cdc7(Δ1–431) and Cdc7(Δ196–372) did not. The deletion mutants GST-Cdc7(Δ279–574) and GST-Cdc7(Δ1–279) also docked at the NPC, albeit at lower levels (Fig. 5C). This result demonstrates that in vitro binding of importin-β to Cdc7 is directly relevant to Cdc7 nuclear transportation in an importin-β-dependent manner.

Further deletion analysis suggested that the Cdc7 segment spanning amino acids 306–313 is important for Cdc7 nuclear localization (data not shown). Therefore, we generated several single and double point mutations within this region, and then examined the nuclear localization of each mutant in transfected CHO cells. As shown in Fig. 5D, Cdc7 K306A and K309A single mutants were localized to the nucleus. However, the Cdc7 K306A,K309A double point mutant was localized in the cytoplasm, suggesting that the Lys306 and Lys309 residues are critical for...
Importin-α2 Competes with Importin-β for Binding to Cdc7 and Can Competitively Inhibit Importin-β-mediated Cdc7 Nuclear Import—Because both importin-α2 and -β bind to the same region of the Cdc7 protein, importin-α2 may compete with importin-β for binding to Cdc7. We therefore carried out a solution binding assay for Cdc7 and importin-β in the presence of different concentrations of importin-α2 protein (Fig. 6A, lanes 2–4). At a molar ratio of 1:1 between importin-α2 and -β, Cdc7 bound to importin-β, but not to importin-α2 (Fig. 6A, lane 2). When the molar ratio of importin-α2 and -β was increased to 5:1, both of them bound to Cdc7 (Fig. 6A, lane 3). Further increase in importin-α2 to a 10:1 molar ratio completely inhibited the binding between Cdc7 and importin-β (Fig. 6A, lane 4). The data shown in Fig. 6A also confirms an earlier observation (Fig. 2C) that importin-α2 and -β can bind to Cdc7 in the absence of the other importin (Fig. 6A, lanes 1 and 6).

The differential binding of Cdc7 to importin-α2 and -β raises the possibility that importin-α2 may inhibit the importin-β-mediated Cdc7 nuclear transportation, in contrast to its classical role of promoting transport of the cargo/importin-α/-β complex into the nucleus. The interference of Cdc7/importin-β complex formation by importin-α2 could be because of a direct interaction of importin-α2 with Cdc7 through the Kinase Insert II domain as predicted by the data shown in Fig. 5. Alternatively, importin-α2 could indirectly disrupt (or interfere) the Cdc7/importin-β complex formation (by binding to importin-β). To determine which of these possibilities is correct, we carried out a binding assay using importin-α2 lacking the IBB domain (ΔIBB: Δ1–51). Like full-length importin-α2, a high level of His-importin-α2ΔIBB (1:10) disrupted the association of GST-Cdc7 with His-importin-β, suggesting that the inhibition of Cdc7 binding to importin-β is due to the direct interactions of importin-α2 to Cdc7 through the Kinase Insert II domain (Figs. 5B and 6A, lane 5).

To further characterize the binding of Cdc7 to importin-α2 and -β, a gel-filtration assay was carried out using a Superdex 200 column (Amersham Biosciences). The mixture of purified recombinant proteins (i.e. His-importin-α2, His-importin-β, and GST-Cdc7) in PBS buffer was loaded onto a column and fractionated by chromatography (Fig. 6B). The protein complexes collected in each fraction were analyzed by Western blotting using either anti-Cdc7 or -His antibodies. Consistent with the data shown in Fig. 6A, the profile of GST-Cdc7 and His-importin-β proteins co-eluted with fractions 2–5, suggesting that Cdc7 primarily forms a complex with importin-β, but not with importin-α2 (Fig. 6B, C and C). Fractions 6–8 contain all three proteins. This raises the possibility that certain complexes may contain all three proteins, Cdc7, importin-α2, and -β. Alternatively, these fractions may contain a mixture of complexes, Cdc7/importin-α2 and Cdc7/importin-β. Because importin-ΔIBB can effectively compete with importin-β for Cdc7, we prefer the latter possibility. This conclusion is also consistent with the expectation that the total molecular weight of protein complexes collected in the fractions would be doublets but not triplets. Fractions 9 and 10 mainly contain Cdc7 and importin-α2, suggesting that Cdc7 can form a complex with importin-α2, which is consistent with the data shown in Fig. 6A (lane 6).

We then determined whether importin-α2 could interfere with importin-β-mediated Cdc7 docking at the NPC. As shown in Fig. 6E, importin-α2 can significantly inhibit importin-β-mediated Cdc7 translocation to the NPC at a molar ratio of 1:5 (βα2) and completely blocked the docking at the 1:10 molar ratio (βα2). This data are consistent with the idea that the interference of the Cdc7/importin-β complex by importin-α2 is directly relevant to the importin-α2-mediated down-regulation of Cdc7 nuclear import.

To further examine the inhibitory effect of importin-α2 on Cdc7 nuclear transportation, we carried out nuclear import assays using HeLa cell cytosol. GST-Cdc7 is imported into the nucleus in the presence of the HeLa cytosol and an energy generating system in the digitonin-permeabilized HeLa (Fig. 7A) and CHO (Fig. 7B) cells. When a low dose of importin-α2 (0.125 mg/ml) was added to the HeLa cytosol, importin-α2 did not notably inhibit Cdc7 nuclear import (Fig. 7A, V and VI). When the amount of importin-α2 was increased to 0.25 mg/ml, Cdc7 nuclear import was almost completely inhibited (Fig. 7A, VII and VIII). Interestingly, the inhibitory effect of importin-α2 was more pronounced in the CHO cells (compared panels V–VIII in Fig. 7, A and B). The reasons for this difference between HeLa and CHO cells are currently unknown.

**DISCUSSION**

We were surprised by the finding that huCdc7 could bind to both importin-α and -β, because huCdc7 does not contain any known CNLS. However, further studies by pull-down and dot-blot analyses confirmed our initial finding. Subsequently, in vitro binding assays carried out using different molar ratios of importin-α and -β led us to conclude that huCdc7, although bound by both importins, has a much higher affinity for importin-β than for importin-α at low protein concentrations. This observation suggests that a similar preferential binding of Cdc7 to importin-β may occur in the cytoplasm, because the low protein concentrations used for our experiments are more relevant to physiological conditions. This may be the reason why Cdc7 can be readily transported into the nucleus despite significant amounts of importin-α in the cyto-
Importin-β-mediated Cdc7 Nuclear Import

plasm under normal cellular conditions. Consistent with this hypothesis, we found that the binding of Cdc7 by importin-β is directly relevant to Cdc7 nuclear import. We first demonstrated that the HeLa cytosol depleted of both importin-α and -β did not support Cdc7 nuclear transportation, which was restored by adding purified recombinant importin-α and -β (Fig. 3B). Importantly, supplementation of importin-β alone to the cytosol depleted of both importin-α and -β could restore Cdc7 nuclear import. In contrast, supplementation of purified importin-α alone to the cytosol depleted of both importin-α and -β did not restore Cdc7 nuclear import. This data strongly suggests that importin-β, but not -α, is responsible for Cdc7 nuclear import. We then repeated the experiment using the HeLa cytosol-depleted importin-α or -β only (Fig. 3C). Depletion of importin-β, but not importin-α, inhibited Cdc7 nuclear localization, and supplementing the depleted cytosol with purified recombinant importin-β restored Cdc7 nuclear import. This data further confirms that importin-β is the sole factor required for the initiation of huCdc7 nuclear import, which was also consistent with the results of in vitro antibody transduction and in vitro docking assays (Figs. 4 and 5).

In vitro binding assays using Cdc7 mutants suggest that the Cdc7 Kinase Insert II (amino acids 203–370) contains binding site(s) for both importin-α and -β (Fig. 5). The mutant Cdc7(D1–146) and wild-type Cdc7 showed a comparable binding affinity for importin-β, whereas the mutant Cdc7(D196–372) did not bind to importin-β at all, suggesting that the Kinase Insert II domain is necessary and sufficient for stable binding to importin-α and -β. Interestingly, both Cdc7(D1–197) and Cdc7(A297–574) could bind to importin-β, albeit at lower affinity, but not to importin-α (Fig. 5). This data raises the following possibilities: 1) there are two separate subregions for importin-β binding, but only one site for importin-α within the Kinase Insert II domain; or 2) there is only one binding site for both importin-α and -β within the entire Kinase Insert II region. If the former is true, one of the two importin-β binding sites would be at amino acids 196–279 and the other at 280–372. Judging from the signal intensities of protein bands in Fig. 5B, importin-β may bind to these two subregions with almost equal probability. If there is only one binding site within the Kinase Insert II domain, it may span both up- and downstream regions of the amino acid residue 279/280 junction. In this case, however, each of the 196–279 and 280–372 protein segments alone may have sufficient binding affinity for importin-β, although both protein segments may be required for more stable binding to importin-β. If the one-binding site scenario is correct, more than one binding motif may be present within the Kinase Insert II domain. At this point, we cannot rule out either possibility, although we prefer the one-binding site model because a double point mutation within a small protein segment could inhibit Cdc7 nuclear localization as discussed below.

Considering that the Kinase Insert II comprises ~170 amino acids, the necessity of this large stretch of protein segment for importin binding is quite different from the cNLS-mediated nuclear transportation. All known proteins transported into the nucleus by importin-β appear to require a large stretch of protein segment (23, 27, 38). These authors suggest that a large binding region is required for importin-β-mediated protein nuclear transportation because the binding of importin-β to a cargo protein is by structural interactions. We, however, found that the Lys306 and Lys309 residues are essential for huCdc7 nuclear localization as the Cdc7 K306A,K309A double point mutant was not localized in the nucleus. This data suggest that the ternary structure of the Cdc7 K306A,K309A mutant has significantly altered so that it can no longer efficiently interact with importin-β. Alternatively, importin-β-mediated protein nuclear localization may also require a specific amino acid sequence (in addition to the structural requirement). In this regard, it may be worth further investigation of the potential role of XXXXXX (which is from 306VKLMKQA of huCdc7) in an importin-β-mediated protein nuclear localization when more proteins directly transported by importin-β are identified and characterized.

The most surprising finding in this study is that importin-α can competitively impede the complex formation of Cdc7 with importin-β, which is directly relevant to the inhibition of Cdc7 nuclear localization (Figs. 6 and 7). Because importin-α inhibits docking of Cdc7 at the NPC in vitro, the inhibition of the Cdc7 nuclear transportation by importin-α is likely at the protein-protein interaction step in the cytoplasm (as opposed to transportation through the NPC). Furthermore, this inhibition of Cdc7 nuclear import by importin-α is independent of importin-α binding to importin-β (Fig. 6).

Our data from dot blot and gel-filtration assays suggest that the affinity between Cdc7 and importin-β is strong when equiproportional concentrations of importin-α and -β are present (α in Fig. 7C). Therefore, the formation of the Cdc7/importin-β complex may readily occur under normal cytoplasmic conditions, resulting in effective Cdc7 nuclear import. For Cdc7 nuclear localization, the two lysine residues at 306 and 309 are critically important (Fig. 5D), perhaps by promoting stable binding between Cdc7 and importin-β as the Cdc7 K306A,K309A mutant shows significantly lower binding affinity for importin-β (Fig. 5E). The binding between Cdc7 and importin-α (β in Fig. 7C) would not normally occur under the equimolar concentration conditions (Fig. 6A). Therefore, importin-α cannot impede Cdc7 nuclear transportation under the normal cytoplasmic conditions. This inability of importin-α binding to Cdc7 may also reduce the association potential of importin-α with importin-β (c of the Fig. 7C), because the IBB of importin-α has a self-inhibitory function (39, 40). If, however, a cargo protein contains a cNLS, this binding dynamic can change dramatically. This is because importin-α can bind to the cargo protein, by which the binding potential of importin-α to -β can also increase through the release of the IBB domain from its cis-inhibitory conformation (39, 40).

It is well known that cells can rapidly activate a replication checkpoint in response to cell damaging agents such as irradiation and anticancer agents (41, 42). Interestingly, Miyamoto et al. (43) have recently found that cellular stress caused by irradiation can induce the nuclear accumulation of importin-α and inhibit a conventional nuclear import. In these contexts, our model predicts an interesting possibility that importin-α may be able to bind to Cdc7 in the nucleus (where no importin-β is present), and is thus involved in the down-regulation of replication initiation when a cell faces a crisis. Because an effective checkpoint operation can increase cell survival, the Cdc7 binding to importin-α in the nucleus in response to cell damage may have significant implication to decreases in the efficacy of anti-cancer therapies.

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