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Role of Importin-β in the Control of Nuclear Envelope Assembly by Ran

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Summary

Compartmentalization of the genetic material into a nucleus bounded by a nuclear envelope (NE) is the hallmark of a eukaryotic cell. The control of NE assembly is poorly understood, but in a cell-free system made from *Xenopus* eggs, NE assembly involves the small GTPase Ran [1, 2]. In this system, Sepharose beads coated with Ran induce the formation of functional NEs in the absence of chromatin [2]. Here, we show that importin-β, an effector of Ran involved in nucleocytoplasmic transport and mitotic spindle assembly, is required for NE assembly induced by Ran. Concentration of importin-β on beads is sufficient to induce NE assembly in *Xenopus* egg extracts. The function of importin-β in NE assembly is disrupted by a mutation that decreases affinity for nucleoporins containing FxFG repeats. By contrast, a truncated protein that cannot interact with importin-α is functional. Thus, importin-β functions in NE assembly by recruiting FxFG nucleoporins rather than by interaction through importin-α with karyophilic proteins carrying classical nuclear localization signals. Importin-β links NE assembly, mitotic spindle assembly, and nucleocytoplasmic transport to regulation by Ran and may coordinate these processes during cell division.

Results and Discussion

Ran GTPase plays roles in nuclear structure and function throughout the cell division cycle [3]. The activity of Ran is determined both by its localization and its guanine nucleotide-bound state. During interphase, Ran is concentrated in the nucleus, mainly in the GTP-bound form, and determines the directionality of nucleocytoplasmic transport by controlling the stability of complexes formed between cargo proteins carrying specific targeting signals and transport proteins. Import complexes formed between proteins with Lys-rich nuclear localization signals (NLS) and importin-β, either directly or through importin-α, are assembled in the cytoplasm and dissociated in the nucleus by the binding of Ran-GTP to importin-β [4]. During mitosis, Ran, mainly GDP bound, is dispersed throughout the cell, while localized generation of Ran-GTP in the vicinity of chromatin releases proteins required for mitotic spindle assembly from inhibitory complexes with importin-β [5–7].

Ran also functions in the assembly of the nuclear envelope (NE) at the end of mitosis when the compartmentalization of the nucleus is reestablished [1, 2, 8]. In cell-free systems for NE assembly made from *Xenopus laevis* eggs, NE assembly requires the generation of Ran-GTP from Ran-GDP by the guanine nucleotide exchange factor RCC1, as well as GTP hydrolysis on Ran [1, 2]. Localized concentration of Ran is sufficient to induce NE assembly in *Xenopus* egg extracts in the absence of chromatin, since Sepharose beads coated with Ran will assemble NE-like structures containing nuclear pore complexes (NPCs) around them, forming pseudonuclei that actively import karyophilic proteins [2]. However, the mechanism by which Ran controls NE assembly is unknown.

To investigate the possible role of Ran-interacting proteins such as importin-β in NE assembly, we depleted *Xenopus* egg extracts using RanQ69L, a mutant defective in GTPase activity and therefore locked in the GTP-bound form [9]. This procedure removed more than 90% of importin-β from the extracts (Figure 1A), as well as other Ran-GTP binding proteins [6]. Extracts depleted of Ran binding proteins (ΔRanBP extracts) were deficient in the ability to promote membrane vesicle recruitment and fusion to form continuous membranes around Sepharose beads coated with Ran (Figure 1B). NE assembly activity in ΔRanBP extracts was restored by the addition of 5 μM importin-β, a concentration similar to that of the endogenous protein in nondepleted extracts [6], but not by a 10-fold excess (50 μM) of importin-β (Figure 1B), which had a dominant inhibitory effect in nondepleted extracts (Figure 2B). By contrast, the related import factors importin-5 and transportin (Trn) were unable to recover NE assembly activity in ΔRanBP extracts, although importin-α and importin-7 weakly promoted vesicle recruitment (Figure 1C).

Similar to full-length importin-β, a truncated protein (importin-β^{1-409}) that lacks importin-α binding activity [10] restored NE assembly around Ran beads in ΔRanBP extracts (Figure 2A), demonstrating that importin-β does not function in NE assembly by interaction through importin-α with karyophilic proteins carrying Lys-rich NLS motifs. By contrast, importin-β^{45-462}, which lacks the Ran binding region [10], was defective in restoring NE assembly activity to ΔRanBP extracts (Figure 2A). Indeed, in nondepleted extracts, 5 μM importin-β^{45-462} (but not

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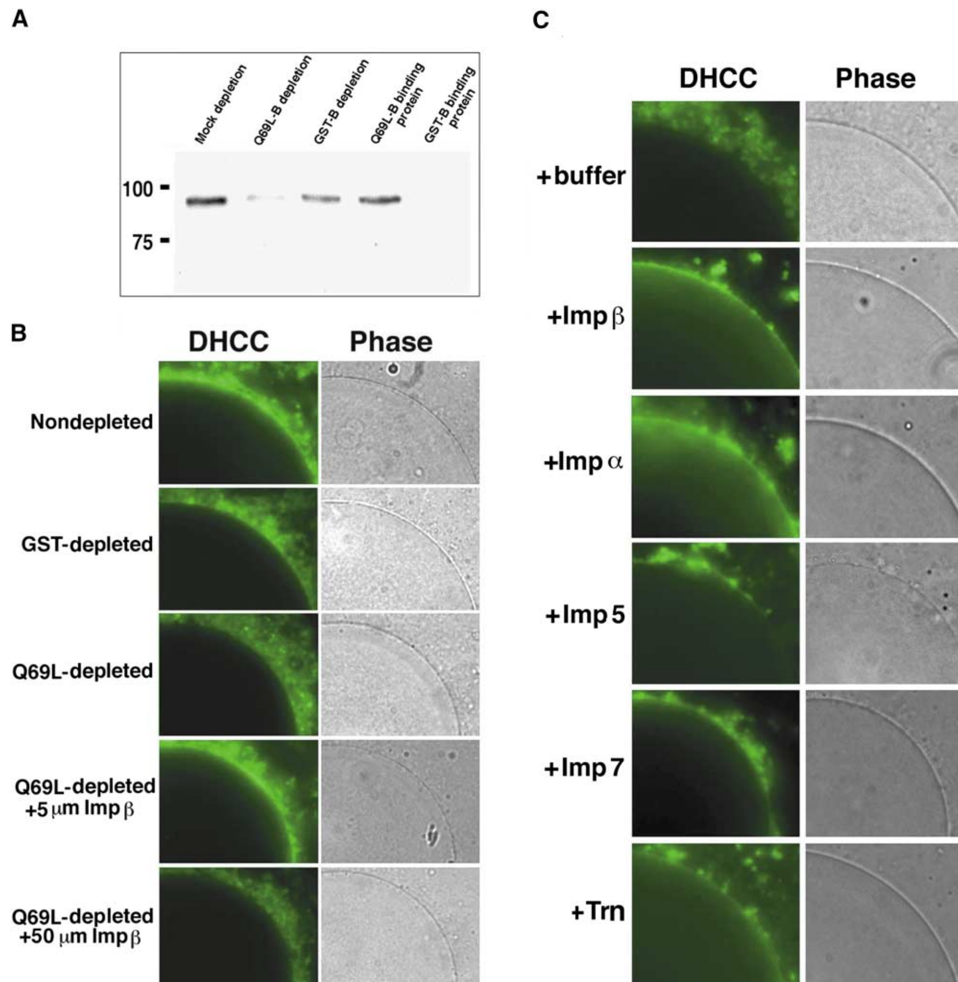

Figure 1. Importin-β Is Required for Nuclear Envelope Assembly Induced by Ran

(A) Depletion of importin-β from *Xenopus* egg extracts using GST-RanQ69L. A Western blot of proteins present in 1 μl depleted extracts or bound to 5 μl washed beads loaded with glutathione-S-transferase (GST) or GST-RanQ69L detected by an antibody to importin-β.

(B) NE assembly on the surface of Sepharose beads coated with Ran incubated in *Xenopus* egg extracts either nondepleted, mock depleted with GST, or depleted with GST-RanQ69L (ΔRanBP extracts). Importin-β (Impβ) was added at the concentration shown. NEs were stained with the lipophilic dye DHCC.

(C) Effect of importin family proteins on NE assembly around Ran beads incubated in ΔRanBP extracts. Importin-β (Impβ), importin-α (Impα), importin-5 (Imp5), importin-7 (Imp7), or transportin (Trn) were added at 5 μM.

5 μM full-length importin-β or importin-β^{1-409}) had a dominant inhibitory effect on NE assembly around Ran beads (Figure 2B). Thus, importin-β is required for NE assembly induced by Ran. These results suggest that importin-β functions as an adaptor that recruits target proteins to Ran during this process.

In addition to transported cargoes and Ran, importin-β interacts directly with protein components of the nuclear pore (nucleoporins) containing FxFG (Phe-x-Phe-Gly, where x is usually Ser, Gly, or Ala) repeats [11]. Both importin-β and nucleoporins were recruited to Ran beads during NE assembly, and this binding was not prevented by the disruption of membrane assembly by the detergent Triton X-100 (see the Supplementary Material available with this article online), suggesting a direct interaction. To test the possible role of importin-β in nucleoporin recruitment, we used proteins in which Ile^{178} is changed to Asp (I178D), which decreases the affinity of importin-β for FxFG nucleoporins but not nucleoporins containing GLFG (Gly-Leu-Phe-Gly) repeats or transport cargoes [11]. This mutation inhibited the ability of full-length importin-β to restore NE assembly activity to ΔRanBP extracts (Figure 2C).

If importin-β serves to recruit FxFG nucleoporins to Ran, then the immobilization and concentration of importin-β might be sufficient to induce NE formation. To test this possibility, we coated Sepharose beads with importin-β proteins and analyzed their ability to induce NE formation in nondepleted *Xenopus* egg extracts. Beads coated with full-length importin-β recruited nucleoporins and membrane vesicles that fused to form a continuous envelope, whereas pretreatment of importin-β with N-ethylmaleimide (NEM), which reacts with cysteine residues on importin-β and inactivates it [12], abolished the ability to induce NE formation (Figure 3A). Importin-β may therefore account, at least in part, for

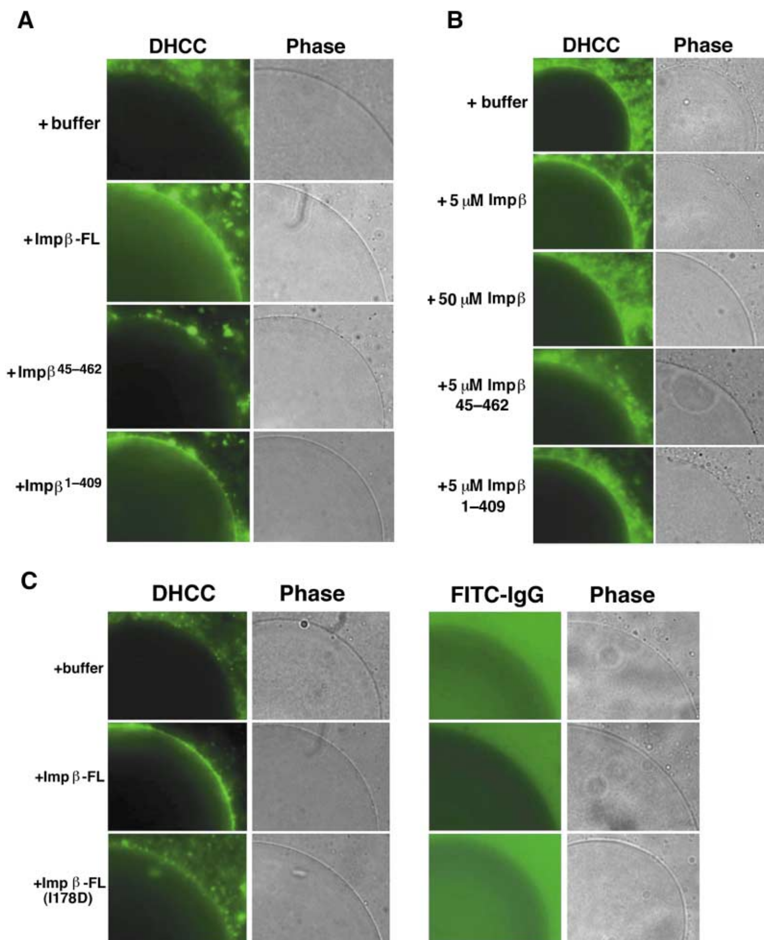

Figure 2. Analysis of the Structural Requirements for Importin-β to Permit NE Assembly around Ran Beads

(A) Full-length importin-β (Impβ-FL) or truncated proteins (Impβ$^{45–462}$, Impβ$^{1–409}$) were added to ΔRanBP extracts.

(B) Dominant inhibitory effects of importin-β proteins on NE assembly in nondepleted extracts.

(C) A mutation that decreases the affinity for nucleoporins (I178D) prevents importin-β from rescuing NE assembly around Ran beads in ΔRanBP extracts. NE assembly was monitored by DHCC staining of membranes and the exclusion of FITC-IgG. Proteins were added at 5 μM.

the NEM sensitivity of NE assembly [13]. Beads coated with importin-β proteins containing the I178D mutation also did not induce NE formation (Figure 3A). Thus, immobilized importin-β, acting through recruitment of FxFG nucleoporins, induces NE assembly. When directly bound to beads, importin-β$^{45–462}$ efficiently induced NE formation (Figure 3A), demonstrating that the interaction of Ran with the immobilized importin-β is not required. Beads coated with importin-α, importin-5, importin-7, or transportin did not induce NE assembly in ΔRanBP extracts (Figure 3B); although, beads coated with importin-7 (Figure 3C) or importin-α (data not shown) were able to form NE when ΔRanBP extracts were supplemented with 5 μM importin-β, but not proteins carrying the I178D mutation (Figure 3C). Both importin-α and importin-7 form complexes with importin-β [14, 15], suggesting that they are able to concentrate importin-β and thereby induce NE assembly.

These results indicate that importin-β plays a role during NE assembly induced by Ran through interaction with FxFG domains on nucleoporins. Considering the complexity of protein components of the NE, multiple FxFG nucleoporins present in either the soluble or vesicle fractions of *Xenopus* extracts may be involved. Binding of Ran-GTP to importin-β reduces the affinity of importin-β for many FxFG nucleoporins [10, 16–18]. Thus, the generation of Ran-GTP by RCC1 concentrated on Ran beads [2, 8] or chromatin [1] may recruit importin-β and release FxFG nucleoporins locally, allowing for the formation of multiprotein complexes that facilitate the recruitment of membrane vesicles. GTP hydrolysis by Ran would permit the recycling of importin-β and the recruitment of further nucleoporins. Ran-GTP may also interact with some nucleoporins directly [19] or through importin-β [10, 20], whereas Ran-GDP can interact with FxFG nucleoporins via NTF2 [21]. Further interactions between Ran, importin-β, and nucleoporins may occur via zinc-finger domains [22, 23] or GLFG repeats [24, 25] on specific nucleoporins. Thus, the complete GDP/GTP cycle on Ran and more than one type of interaction might be required to recruit the full compliment of nucleoporins for nuclear pore complex (NPC) assembly.

Ran controls NE formation through the same primary effector molecule, importin-β, that controls mitotic spindle assembly during mitosis [5–7] and nuclear import during interphase [4]. At the end of mitosis, relocalization of Ran to chromatin [26] and the concentration of importin-β could recruit nucleoporins while inhibiting microtubule-stabilizing factors in the vicinity of chromatin. Thus, changes in the localization, concentration, and molecular interactions of Ran and importin-β may help coordinate disassembly of the mitotic spindle with reassembly of the NE and the restarting of nucleocytoplasmic transport.

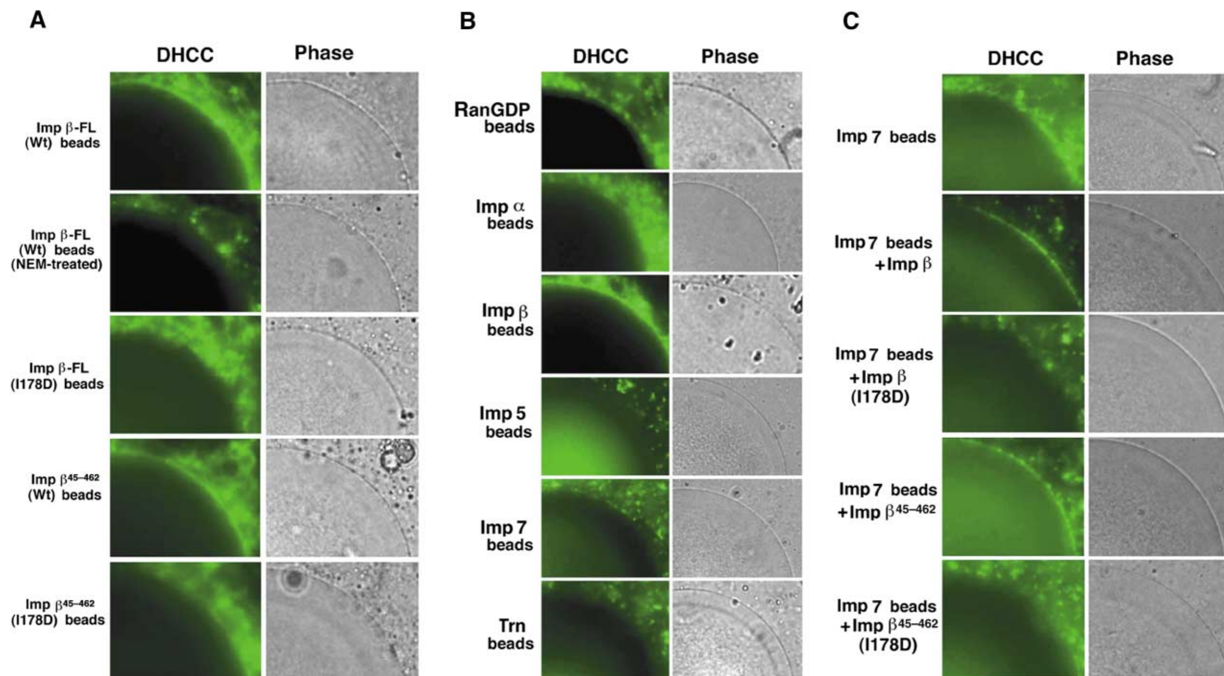

Figure 3. Immobilized Importin-β Induces NE Assembly

(A) Beads coated with importin-β full-length (FL) and truncated proteins incubated in nondepleted extracts.

(B) Beads coated with importin-β and related proteins incubated in ΔRanBP extracts.

(C) Beads coated with importin-7 incubated in ΔRanBP extracts with the addition of 5 μM importin-β proteins.

Experimental Procedures

Recombinant Proteins

Recombinant human Ran proteins were prepared as glutathione-S-transferase (GST) fusions in *Escherichia coli*, purified, and loaded with nucleotides as described previously [27]. Wild-type Ran was loaded with GDP, and RanQ69L was loaded with GTP. Importin-β, including mutants, importin-α, importin-5, importin-7, and transportin were prepared as described previously [10] in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, and 250 mM sucrose.

Xenopus Egg Extracts

Xenopus laevis egg extracts were prepared by the method of Hutchison [28], frozen, and stored in aliquots in liquid nitrogen. To deplete extracts of Ran binding proteins, 100 μl extract was mixed with an equal volume of packed glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) prebound with GST-RanQ69L loaded with GTP. The extract was incubated with mixing at 4°C for 60 min, and the beads were pelleted by centrifugation. The supernatant was subjected to this procedure three times to produce Ran binding protein-depleted (ΔRanBP) extract. Mock-depleted extracts were treated in the same way using glutathione-Sepaharose 4B beads with bound GST.

Nuclear Envelope Assembly

Nuclear envelope assembly around glutathione-Sepharose 4B beads coated with GST-Ran was carried out as described previously [2], except that beads were washed in KHM buffer (78 mM KCl, 50 mM HEPES (pH 7.0), 4 mM $MgCl_2$, 2 mM EGTA, and 1 mM dithiothreitol) before the addition to extracts. The beads have a diameter of approximately 100 μm. Importins were coupled directly to NHS-Sepharose (Amersham Pharmacia Biotech). After incubation at 23°C for 30 min in *Xenopus* egg extracts supplemented with an ATP-regenerating system and cycloheximide [28], samples were removed and stained on a slide with 3,3′-dihexyloxacarbocyanine (DHCC) without fixation. In some instances, NE integrity was assessed after incubation for 60 min with the addition of FITC-IgG, which is too large to diffuse across nuclear pores and is excluded by an intact NE. In each case, phase optics were used to focus before capturing fluorescence images. For immunofluorescence, samples were fixed for 30 min in 4% formaldehyde (in 10 mM PIPES (pH 7.2), 80 mM KCl, 5 mM EDTA, and 15 mM NaCl). Beads were then recovered onto coverslips, permeabilized with 0.1% Tritron X-100 for 5 min, and prepared for immunofluorescence microscopy using specific primary antibodies against nucleoporins (mAb414, 1:500) or importin-β (Transduction Laboratories, 1:100). Images were captured immediately on a Zeiss Axioskop microscope using a cooled charged-coupled device camera (Hamamatsu Photonics) and were processed with Improvision Openlab and Adobe PhotoShop software. For Western blotting, beads were recovered from *Xenopus* egg extracts, and bound proteins were separated on polyacrylamide gels, transferred to nitrocellulose, and developed by chemiluminecence as described previously [2] using the same primary antibodies used for immunofluorescence.

Supplementary Material

Supplementary Material including figures showing that Sepharose beads coated with Ran recruit FxFG nucleoporins and importin-β when incubated with *Xenopus* egg extracts and beads coated with Ran or importin-β form intact nuclear envelopes that exclude a fluorescent IgG is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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