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Importin β Is a Mitotic Target of the Small GTPase Ran in Spindle Assembly

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Summary

The GTPase Ran has recently been shown to stimulate microtubule polymerization in mitotic extracts, but its mode of action is not understood. Here we show that the mitotic role of Ran is largely mediated by the nuclear transport factor importin β. Importin β inhibits spindle formation in vitro and in vivo and sequesters an aster promoting activity (APA) that consists of multiple, independent factors. One component of APA is the microtubule-associated protein NuMA. NuMA and other APA components are discharged from importin β by RanGTP and induce spindle-like structures in the absence of centrosomes, chromatin, or Ran. We propose that RanGTP functions in mitosis as in interphase by locally releasing cargoes from transport factors. In mitosis, this promotes spindle assembly by organizing microtubules in the vicinity of chromosomes.

Introduction

During cell division, a complete set of duplicated chromosomes must be evenly distributed to the two daughter cells. The cellular structure that performs this task is the mitotic spindle, a macromolecular complex consisting primarily of microtubules and associated proteins (Desai and Mitchison, 1997). At the onset of mitosis, activation of cdc2 kinase leads to an increase in microtubule dynamics and the interphase microtubule array is dismantled. The minus ends of microtubules are focused into two poles, while the plus ends are oriented toward chromosomes, creating the typical bipolar spindle (Hyman and Karsenti, 1996). In most animal cells, microtubule organization is strongly influenced by the centrosome (for review see Andersen, 1999). However, bipolar spindles also assemble in female meiotic cells that lack centrosomes (Gard, 1992; Matthies et al., 1996), in somatic cells in which the centrosome has been inactivated (Khodjakov et al., 2000), or around chromatin-coated beads in the absence of centrosomes or kinetochores (Heald et al., 1996).

Despite apparent differences in spindle assembly pathways, two unifying mechanisms appear to operate in all systems. One is the role of microtubule-based motor proteins, including cytoplasmic dynein and a family of kinesins, which are localized to the spindle and function to cross-link and move microtubules or chromosomes (Heald, 2000). The second principle is the stabilizing force of chromosomes, which generates the anisotropy of microtubule growth and attachment necessary for spindle formation. One stabilizing force consists of kinetochores, which capture microtubule plus ends (Kirschner and Mitchison, 1986; Huitorel and Kirschner, 1988). In addition, evidence exists for another mechanism driving spindle assembly, the local stabilization of microtubules by mitotic chromosome arms or chromatin (Nicklas and Gordon, 1985; Dogterom et al., 1996; Heald et al., 1996; Andersen et al., 1997). The mechanism behind microtubule stabilization by chromatin is not understood, but a candidate for the mediator of the chromatin-microtubule signal has recently been identified as the small GTPase Ran (reviewed in Azuma and Dasso, 2000; Heald and Weis, 2000).

Ran, like other small GTP binding proteins, cycles between a GDP and a GTP bound state and this cycle is accelerated by a guanine nucleotide exchange factor (GEF) (Bischoff and Ponstingl, 1991) and by a GTPase activating protein (GAP) (Bischoff et al., 1994). One hallmark of the Ran cycle is the compartmentalization of the regulatory proteins. Ran's GEF, RCC1, is bound to chromatin and thus is nuclear during interphase (Ohtsubo et al., 1989), whereas the RanGAP is confined to the cytoplasm (Matunis et al., 1996; Mahajan et al., 1997). Therefore, it is predicted that RanGTP is highly enriched in the nucleoplasm of interphase cells and that there is a steep RanGTP/RanGDP gradient between the nucleus and the cytoplasm (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999).

The best characterized role of Ran is in macromolecular transport into and out of the nucleus, where Ran functions together with a large family of soluble receptor proteins named importins or exportins (Mattaj and Englmeier, 1998; Weis, 1998). Importins recognize their substrates in the cytoplasm and transport them through nuclear pores into the nucleus. In the nucleoplasm, RanGTP binds to importins and induces the release of import cargoes (Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). This pathway is exemplified by importin β, which mediates the import of a large variety of cargo molecules including proteins containing classical nuclear localization signals (NLSs) (reviewed in Koepp and Silver, 1998), snRNPs (Palacios et al., 1997; Huber et al., 1998), and several ribosomal proteins (Jäkel and Görlich, 1998). Importin β is able to bind to its import substrates either directly or indirectly with the help of adaptor proteins such as importin α, which is responsible for the recognition of NLS-containing proteins (reviewed in Weis, 1998; Görlich and Kutay, 1999).

Exportins interact with their substrates only in the presence of RanGTP. After assembly in the nucleus, the trimeric exportin/RanGTP/cargo complex is translocated to the cytoplasm. Cargo delivery is completed by

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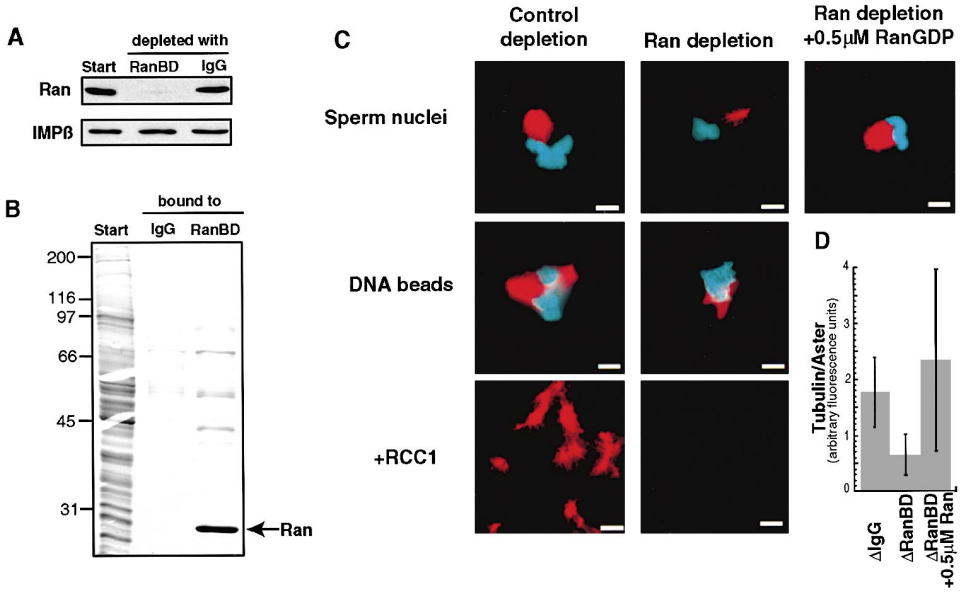

Figure 1. Ran Is Necessary for Spindle Assembly in *Xenopus* Egg Extract

(A) Extract depleted with the Ran binding domain of importin β [aa 1–380] or human IgG was analyzed by Western blot. Membranes were probed with antibodies against Ran and importin β. More than 99% of Ran is removed after passage over the Ran binding domain (RanBD) matrix.

(B) Starting extract and eluates from the IgG and importin β [1–380] RanBD matrices were analyzed by SDS-PAGE followed by Coomassie staining. Load in the bound fractions corresponds to 250× the starting material.

(C) Microtubule structures do not assemble around chromatin in the absence of Ran. The fluorescent signal of the Texas-red tubulin appears red and DNA stained with Hoechst is blue. Upper panels: demembranated sperm nuclei were added to extracts depleted either with IgG beads (left), RanBD beads (center), or depleted with the RanBD beads and supplemented with 0.5 μM of recombinant Ran loaded with GDP (right). Middle panel: chromatin beads were incubated in control or Ran-depleted extracts. Lower panel: 12 μM of recombinant untagged human RCC1 was added to each extract. All reactions were allowed to proceed for 25 min. at 20°C. No polarized microtubule growth was observed after 1 hr of incubation of sperm nuclei in the Ran-depleted extract (data not shown). Bar, 10 μm.

(D) Quantitation of microtubule fluorescence intensity in the presence of sperm nuclei. 30 to 50 nuclei-associated asters were quantified and averaged for each reaction. The error bars represent aster to aster standard deviation.

RanGTP hydrolysis, which is induced by RanGAP and RanBP1 and causes disassembly of the export complex (Koepp and Silver, 1998; Mattaj and Englmeier, 1998; Weis, 1998; Görlich and Kutay, 1999). Members of the exportin family include CRM1, CAS, and exportin-t (reviewed in Weis, 1998). The general function of Ran in import and export is therefore to regulate the compartment-specific binding and release of transport substrates, and Ran confers directionality to this nucleocytoplasmic transfer (reviewed in Nachury and Weis, 1999; Görlich and Kutay, 1999).

An additional role for Ran in mitosis is suggested by several recent studies. Immunodepletion of RCC1 from mitotic *Xenopus* egg extracts inhibited microtubule polymerization and prevented spindle assembly around sperm nuclei (Ohba et al., 1999). Furthermore, addition of excess RanGTP caused spontaneous microtubule polymerization and formation of asters and spindle-like structures in the absence of centrosomes or chromosomes (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). These results suggest that RanGTP, which is generated by chromatin bound RCC1, signals microtubule polymerization specifically in the vicinity of chromosomes (Carazo-Salas et al., 1999; Kalab et al., 1999; reviewed in Heald and Weis, 2000). However, it is not understood how RanGTP regulates microtubule nucleation or stability.

The aim of this study was to identify the targets of Ran in mitotic spindle assembly. We show here that mitotic effects of Ran are mediated by the transport factor importin β. Importin β acts as an inhibitor of spindle assembly in vitro and in vivo by sequestering a microtubule aster promoting activity collectively termed APA. We identify the nuclear mitotic apparatus protein (NuMA) as one component of APA. RanGTP indirectly promotes microtubule polymerization by inducing the localized release of APA from importin β. Therefore, Ran functions in mitosis as in interphase by regulating the compartmentalized release of cargoes from transport factors.

Results

Ran Is Required for Spindle Assembly

Recently, several groups have reported that high concentrations of RanGTP are sufficient to induce the ectopic formation of microtubule asters and spindle-like structures in mitotic extracts derived from *Xenopus* eggs (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). To test whether Ran is necessary for spindle assembly, we took advantage of the amino-terminal Ran binding domain of importin β-like proteins to deplete Ran from *Xenopus* egg extracts (Figure 1). One round of depletion removed more than 99% of Ran from the extract,

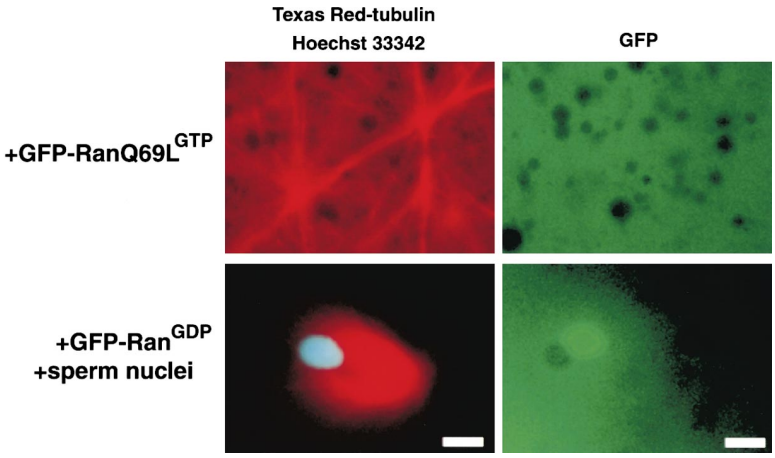

Figure 2. Ran Does Not Colocalize with Microtubules

Formation of asters was followed by fluorescence microscopy after addition of 25 μM GFP-RanQ69L to the egg extract (top row). Images were captured 20 min after starting the reaction (40× objective). Microtubules were polymerized to a similar extent as in the presence of 25 μM RanQ69L, indicating that the GFP-fusion protein is functional. In a separate experiment, sperm nuclei were added to *Xenopus* egg extract together with 1 μg/ml of DNA dye Hoechst and 0.5 μM GFP-Ran loaded with GDP (bottom row). Images were captured after 20 min of incubation (40× objective).

whereas Ran levels remained unaffected in control depletion reactions (Figure 1A). The Ran depletion was specific since no other major proteins coprecipitated (Figure 1B). When Ran-depleted extracts were assayed for spindle formation in the presence of demembranated sperm nuclei, spindle assembly was severely inhibited compared to reactions with control extracts (Figures 1C and 1D). Spindle formation could be restored by the addition of recombinant Ran demonstrating that Ran was the only active component removed by this approach. The Ran-depleted extract was also used to analyze microtubule polymerization induced by either chromatin beads, or by high concentrations of RCC1 in the absence of chromatin (Figure 1C). Again, the formation of spindles and spindle-like structures was greatly impaired after Ran depletion, and RCC1 was no longer able to induce the formation of ectopic microtubule asters. These results suggest that Ran functions downstream of RCC1 and that Ran is necessary for spindle formation in *Xenopus* egg extracts.

Ran Affects Microtubule Dynamics through an Inhibitory Ran Binding Protein

Ran could either influence microtubule dynamics directly or regulate microtubule polymerization through additional downstream factors. To test whether Ran binds to or is enriched on microtubules, we visualized Ran during aster and spindle assembly reactions (Figure 2). The hydrolysis-deficient RanQ69L mutant and wild-type Ran were expressed as GFP fusion proteins and added to *Xenopus* egg extracts. When spindle assembly was induced by the addition of sperm nuclei, GFP-Ran was found to be 2- to 3-fold enriched on condensed chromatin. However, no overlap was detected between the Ran signal and microtubules during the course of spindle assembly, or during the formation of GFP-RanQ69L-induced asters (Figure 2 and data not shown). This result implies that Ran does not stably interact with microtubules and suggests that RanGTP regulates microtubule dynamics indirectly through additional factors.

In order to identify the factor(s) that function downstream of Ran in mitosis, we sought to remove all RanGTP binding proteins (RanBPs) from extracts and analyze the effects of this depletion on microtubule polymerization (Figure 3). RanQ69L bound to GTP was used to affinity deplete *Xenopus* egg extracts. To examine the specificity and efficiency of the approach, the depleted extract (ΔRanBP extract) and a mock-treated extract were compared using antibodies against known Ran binding proteins (Figure 3A). All the members of the importin β superfamily tested here, including importin β itself and the exportins CAS and CRM1, were removed by more than 90% from the extract. In addition, more than 99% of RanBP1 and RanGAP, and to a lesser extent RCC1, were depleted by the incubation with RanQ69L beads. The levels of endogenous Ran remained unaffected by the procedure.

When assayed for spindle assembly, the mock-treated control extract rapidly promoted the formation of ectopic asters following RanQ69L-GTP addition but, as expected, did not form any microtubule-containing structures in the absence of RanQ69L (Figure 3B, upper panel). In contrast, even in the absence of exogenous RanGTP, spontaneous aster formation was seen in the ΔRanBP extract and abundant microtubule figures were detected (Figure 3B, lower panel left). Addition of RanQ69L did not further increase the number or size of these microtubule-containing structures (Figure 3B, lower panel right). On average 55 asters were seen per 60× field in the ΔRanBP extract without addition of RanQ69L. This is comparable with the maximum number of figures observed after the addition of 15 μM RanQ69L-GTP in control extracts (Figure 3B, upper panel right). One explanation for this surprising result is that the RanQ69L matrix removed an activity that inhibits spontaneous microtubule polymerization. However, the experiment did not rule out that removal of RanBPs and RanGAP stabilized Ran in the GTP bound form and that endogenous RanGTP was responsible for the observed aster formation.

To differentiate between these two possibilities, endogenous Ran was removed from the ΔRanBP extract using the affinity-depletion method described above. As shown in Figure 1, the removal of Ran renders untreated extracts insensitive to high concentrations of RCC1 and inhibits spindle formation in the presence of chromatin. The double-depleted extract in which both Ran and RanBPs were removed (ΔRanΔRanBP) still formed ectopic microtubule asters without the addition of chroma-

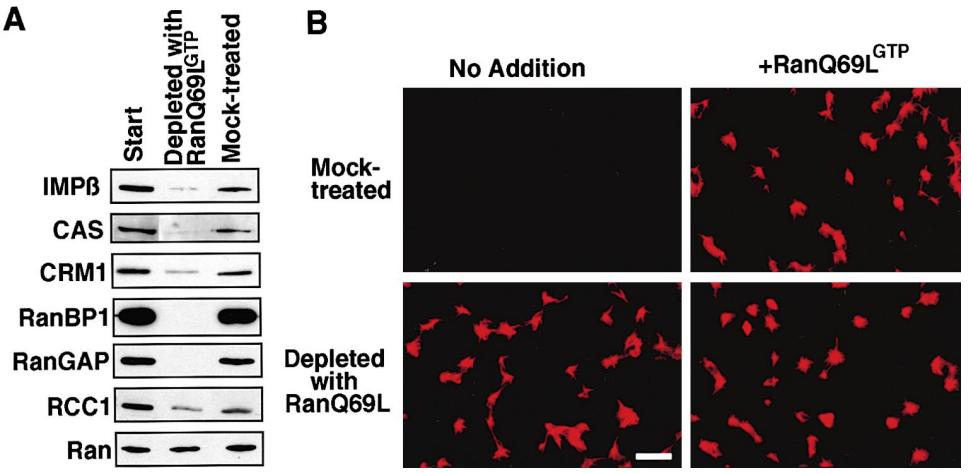

Figure 3. The Depletion of RanGTP Binding Proteins from *Xenopus* Egg Extract Leads to De Novo Microtubule Polymerization

(A) Western blot analysis of the presence of transport factors and Ran cycle regulators in untreated extract, extract depleted by RanQ69L, or mock-depleted extract. (B) Mock-treated extracts or extracts depleted with RanQ69L were incubated for 20 min at 20°C either in the absence (right) or the presence of 15 μM RanQ69L (left). Reactions were then fixed and spun onto coverslips.

tin, RCC1, or RanGTP (data not shown but see quantitation of the result in Figure 6A, column IMPβ [1–380]). Thus, Ran is no longer required for the formation of microtubule structures in ΔRanBP extracts. These results are consistent with the conclusion that *Xenopus* egg extracts contain an inhibitory activity that prevents spindle or aster formation. This inhibitor is inactivated by the addition of RanGTP or removed by RanQ69L beads.

Importin β Acts as a Specific Inhibitor of Spindle Formation

Since the inhibitory activity could be removed by a RanGTP matrix, a number of known RanGTP binding proteins were tested for their ability to inhibit spontaneous aster formation in ΔRanBP extracts (Figure 4A). Again, no microtubule structures could be observed in mock-depleted control extracts, yet, ~55 asters per 60× field were formed spontaneously when the ΔRanBP extract was incubated at 20°C. An equivalent number of microtubule-containing figures was seen after the addition of 4 μM recombinant transportin 1, importin 5, CRM1, or CAS to the ΔRanBP extract. This concentration is comparable to the estimated endogenous concentration of these factors in *Xenopus* extracts (Ribbeck et al., 1998). In contrast, addition of the same amount of importin β reduced the number of asters dramatically and only 4–7 asters were observed per 60× field.

Importin β contains three functionally separable domains required for its interaction with RanGTP, components of the nuclear pore complex, and cargoes (Chi et al., 1997; Kutay et al., 1997). As aster formation in ΔRanBP extracts was independent of Ran, it was of interest to determine which domain of importin β was responsible for the observed inhibition. Full-length importin β, the RanGTP binding domain (importin β [1–380]), and the cargo binding domain (importin β [71–876]) were purified from *E. coli* and added to ΔRanBP extracts (Figure 4B). As shown before, addition of full-length importin β greatly diminished the number of microtubule asters. Only slight inhibition was seen when importin β [1–380] was included in the assay. However, addition of the carboxy-terminal cargo binding domain, importin β [71–876], led to a severe reduction in the number of microtubule-containing figures. When added at equimolar concentration, importin β [71–876] was an even more potent inhibitor of aster formation than full-length importin β.

Next, we analyzed the functional effects of importin β on spindle assembly in the presence of chromatin beads or demembranated sperm nuclei (Figure 4C). Equimolar amounts of full-length importin β, importin β [1–380], and importin β [71–876] were added to extracts and spindle formation was monitored. Full-length importin β and importin β [71–876] severely inhibited the assembly of mitotic spindles in both assay systems, whereas addition of importin β [1–380] had no effect at this concentration (Figure 4C). This confirms the inhibitory function of the cargo binding domain of importin β and suggests that importin β is required to mediate the effects of mitotic chromatin on spindle assembly.

The Mitotic Role of Importin β Is Conserved in Mammalian Somatic Cells

To address whether importin β also performs a function during mitosis in mammalian somatic cells, we microinjected truncated versions of importin β into PtK1 cells. To prevent secondary effects by interfering with nucleocytoplasmic transport, cells were microinjected during late prophase or during prometaphase and then fixed 40–60 min later. X-rhodamine-labeled tubulin was coinjected to allow visualization of the microtubule cytoskeleton (Figure 5). Injecting the Ran binding domain of importin β, importin β [1–380], did not perturb mitotic spindle assembly, and 14 out of 15 cells (93%) injected with this construct proceeded normally through mitosis with 7% of these in metaphase and 93% displaying segregated chromosomes and having progressed to anaphase or telophase (Figures 5A and 5B). These cells were similar in appearance and phase of mitosis to those

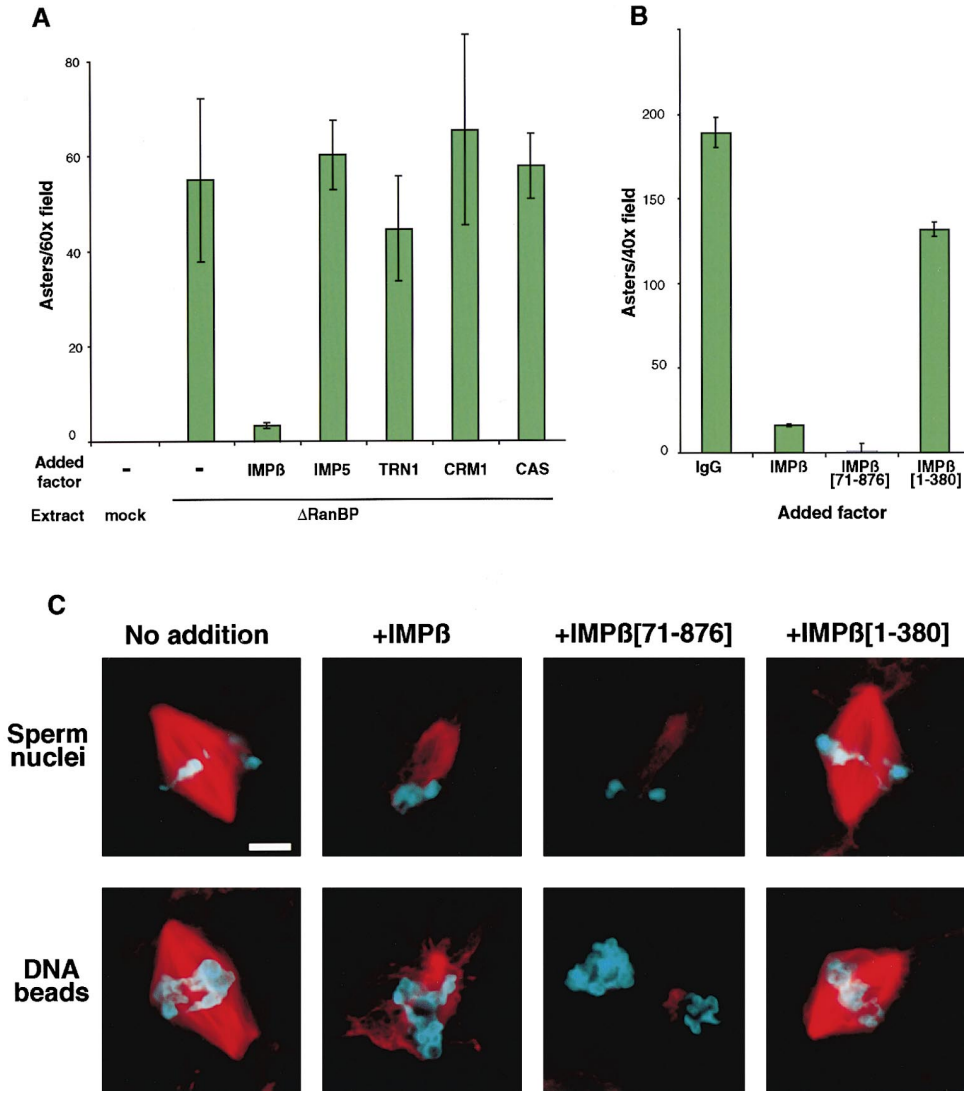

Figure 4. Importin β Inhibits Microtubule Stabilization through Its Cargo Binding Domain

(A) Recombinant transport factors were added at a concentration of 4 μM to ΔRanBP extracts and incubated for 25 min at 20°C. Asters were counted in ten randomly selected fields (60× objective). The error bar represents the field-to-field standard deviation. (B) 4 μM of human IgG, importin β, importin β [71–876] or 16 μM of importin β [1–380] were added to the ΔRanBP extract. Aster reactions were allowed to proceed for 25 min at 20°C. (C) Sperm spindle reactions (upper panel) and DNA bead spindle reactions (lower panel) were performed in egg extracts alone or in the presence of 6 μM importin β, importin β [71–876], or importin β [1–380]. The presence of importin β [71–876] severely inhibited spindle formation in both assay systems. Note that under these conditions, microtubules were only observed around very large aggregates of DNA beads such as the example shown here (lower panel IMP β [71–876]). Smaller bead clusters had no associated microtubules (data not shown).

injected with labeled tubulin alone (data not shown). In contrast, injecting the cargo binding domain, importin β [71–876], had a profound effect on microtubule morphology and progression through mitosis, as only 4 out of 26 cells (15%) possessed normal mitotic figures, 7.5% of these in metaphase and only 7.5% properly segregating their chromosomes and proceeding to anaphase and telophase. A high proportion of the injected cells (62%) possessed highly disorganized microtubule arrays that completely failed to align and segregate chromosomes (Figures 5C and 5D). Although structures corresponding to spindle poles could sometimes be distinguished, microtubules did not grow in a polarized fashion around chromosomes. Instead, microtubules were often bundled, extremely short, or radiating away from the chromosomes (Figures 5C and 5D). An additional six cells (23%) possessed normal microtubule morphology, but displayed defects in spindle structure and/or chromosome segregation (Figure 5E). In addition to spindle and chromosome segregation defects, over 65% of cells injected with importin β [71–876] possessed extremely aberrant chromosomes with a decondensed and fenestrated morphology, indicating other possible mitotic roles for importin β. Therefore, importin β appears to perform a mitotic function in somatic cells similar to its role in *Xenopus* egg extracts.

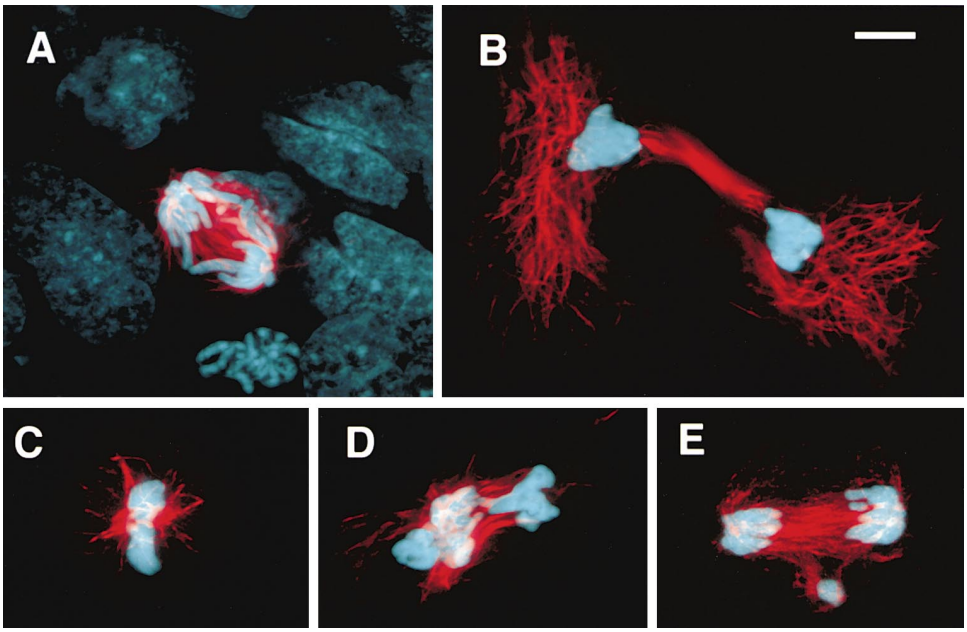

Figure 5. Mitotic Function of Importin β Is Conserved in Mammalian Somatic Cells

Cells were injected in prophase or prometaphase with 10 μM X-rhodamine-labeled tubulin plus importin β constructs and then fixed 30–60 min later. (A and B) Injection buffer contained 650 μM importin β [1–380]. Cell in (A) progressed normally into anaphase and cell in (B) to telophase. (C–E) Injection buffer contained 330 μM importin β [71–896]. (C and D) Examples of cells with highly abnormal microtubule morphologies that failed to form a spindle or properly segregate chromosomes (62% of injected cells). Note decondensed and fenestrated chromosomes (65% of injected cells). (E) Example of cell with normal microtubule morphology but chromosome segregation defect likely to result in aneuploidy (23% of injected cells). Scale = 10 μm.

Importin β Sequesters Multiple Aster Promoting Activities (APAs)

Importin β binds to its cargoes in the absence of Ran, and binding to RanGTP induces the localized release of substrates from importin β. Based on the results described above, it appeared likely that importin β inhibits microtubule polymerization by sequestering an activity that stabilizes microtubules and induces aster formation. This aster promoting activity (APA) would be activated by RanGTP-triggered release from importin β. To examine this model, the ΔRanBP extract was affinity-depleted with the importin β truncations used in the experiments described in Figures 4 and 5. Full-length importin β, importin β [1–380], and importin β [71–876] were immobilized and used to deplete ΔRanBP extracts (Figure 6A). Consistent with the inhibition experiments shown in Figures 4 and 5, importin β [71–876] and, to a lesser extent, also full-length importin β were able to deplete APA from the ΔRanBP extract. Only a minor effect was seen with an importin β [1–380] matrix confirming that the inhibitory effect of importin β is specifically mediated by its cargo binding domain.

A major cargo of importin β is importin α, which functions as an adaptor between importin β and NLS-containing proteins (Görlich et al., 1996; Weis et al., 1996a). The interaction between importin α and importin β requires the intact carboxyl terminus of importin β and the amino-terminal importin β binding (IBB) domain of importin α (Görlich et al., 1996; Weis et al., 1996a; Chi et al., 1997; Kutay et al., 1997). To test whether importin α mediates the effects of importin β in aster formation, we examined if the IBB domain could competitively discharge APA from importin β and induce aster formation in nontreated extracts. The addition of 30 μM IBB to *Xenopus* egg extracts induced the spontaneous formation of aster-like microtubule figures (Figure 6B). Importantly, IBB-induced aster formation could be inhibited by the addition of the NLS binding domain of importin α (Figure 6B). Since this importin α fragment does not interact with importin β, this result suggests that the IBB effect is mediated by a cargo of importin α.

The microtubule asters observed after the addition of saturating amounts of IBB were fewer in number and larger than the ones seen after incubation in the presence of RanQ69L-GTP. Moreover, IBB-induced aster formation could be further stimulated by the addition of RanQ69L-GTP (Figure 6B). We therefore suspected that additional APAs are present in the ΔRanBP extract. To directly analyze whether importin α is the only mediator of APA function, we used an importin β truncation (importin β [1–601]), which no longer binds to importin α but is able to interact with other characterized cargoes (Jäkel and Görlich, 1998; M. V. N. and K. W., unpublished data). Depletion with an importin β [1–601] matrix inhibited the formation of ectopic asters (Figure 6A) but did not remove importin α from the extract (Figure 6C). We conclude that there is also an importin α-independent activity in the ΔRanBP extracts that is distinct from the importin α-dependent activity activated by the IBB addition.

NuMA Is One Component of APA

In order to identify the molecular nature of APA, we sought to restore spontaneous aster formation in extracts that had been depleted with both RanQ69L and importin β [71–876] beads (ΔRanBPΔAPA extract). Our

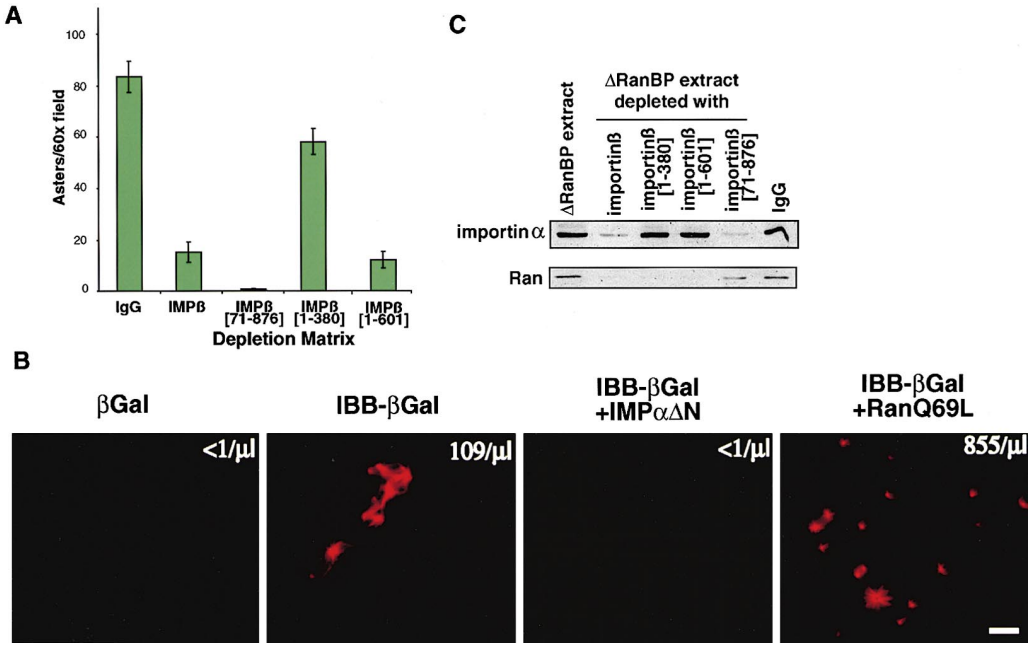

Figure 6. Importin β Functions by Sequestering Multiple Aster Promoting Activities

(A) The extract depleted by RanQ69L was combined with beads coated with human IgG, importin β, importin β [71–876], importin β [1–380], or importin β [1–601]. The supernatant was collected and assayed for spontaneous aster formation. (B) Competitive release of importin β cargoes induces spontaneous aster formation. Extracts were incubated in the presence of either 30 μM βGal, 30 μM IBB-βGal, 30 μM IBB-βGal + 30 μM human importin α1-ΔN65, or 30 μM IBB-βGal + 40 μM RanQ69L. Microtubule-containing structures were counted and averaged to the reaction volume. (C) Western blot of the depleted extracts probed for importin α and Ran. Importin β and importin β [71–876] depleted importin α with ~90% efficiency whereas importin β [1–380], importin β [1–601], and IgG had no effect. In contrast, importin β, importin β [1–380], and importin β [1–601] matrices depleted Ran with a 99% efficiency whereas importin β [71–876] and IgG columns did not remove Ran. Note: immobilized transportin was also able to deplete Ran to a similar level without affecting aster formation (data not shown).

results predicted that APA is present in an activated/free form in ΔRanBP extracts and binds either directly or indirectly to importin β. We therefore tested whether addition of the ΔRanBP extract or an eluate from an importin β column reconstituted aster formation in the ΔRanBPΔAPA extract (Figure 7A). Whereas no microtubule asters were seen in the ΔRanBPΔAPA extract alone or in the presence of RanQ69L-GTP, microtubule-containing figures were readily detected when eluates from the importin β beads or a 1:20 dilution of the ΔRanBP extract were added to the ΔRanBPΔAPA extract (Figure 7A). In addition, we were able to induce microtubule asters when purified microtubule-associated proteins (MAPs; see Experimental Procedures) were added to the ΔRanBPΔAPA extract (Figure 7A). Addition of the same amount of the MAP fraction or the ΔRanBP extract had no effect in untreated extracts (data not shown). Thus the double depleted ΔRanBPΔAPA extract was still capable of forming microtubule asters. Aster formation was restored by an activity that can be eluted from the importin β affinity matrix and is present in an active form in the ΔRanBP extract. In addition, at least one component of APA is present in a purified MAP fraction.

A good candidate for an APA component is the nuclear mitotic apparatus protein (NuMA), a MAP important for microtubule organization in the spindle (Gaglio et al., 1995; Merdes et al., 1996). NuMA is nuclear in interphase and contains a bipartite nuclear localization signal in its carboxyl terminus (Lydersen and Pettijohn, 1980; Tang et al., 1994; Gueth-Hallonet et al., 1996; Saredi et al., 1996). Since NuMA is present in the MAP fraction and was found to be specifically enriched in the eluates of the importin β column (data not shown), we tested whether recombinant NuMA is able to interact with importin β. The carboxyl terminus of NuMA (NuMA tail II; Merdes et al., 1996) bound to importin β, but only in the presence of importin α (Figure 7B). This interaction was abolished by addition of RanGTP (Figure 7B). This result indicates that NuMA is an importin β cargo regulated by RanGTP, and as with other NLS-containing proteins, the interaction is mediated by importin α.

The NuMA tail II domain has been shown to possess microtubule binding activity, and at high concentrations (7 μM) can induce aster formation in egg extracts (Merdes et al., 1996). To test directly whether NuMA contributes to APA, the tail II domain was added to double depleted ΔRanBPΔAPA extracts (Figure 7C). Increasing amounts of the protein induced the formation of microtubule-containing asters in the depleted extract, with a maximum of 12 asters per μl when 400 nM NuMA was present, a concentration that had no effect in undepleted extracts (Figure 7C). Taken together, these data provide strong evidence that NuMA is a part of the aster promoting activity that is sequestered by importin β and discharged in the presence of RanGTP.

Discussion

The polarized organization of the mitotic spindle is essential for proper chromosome segregation during cell

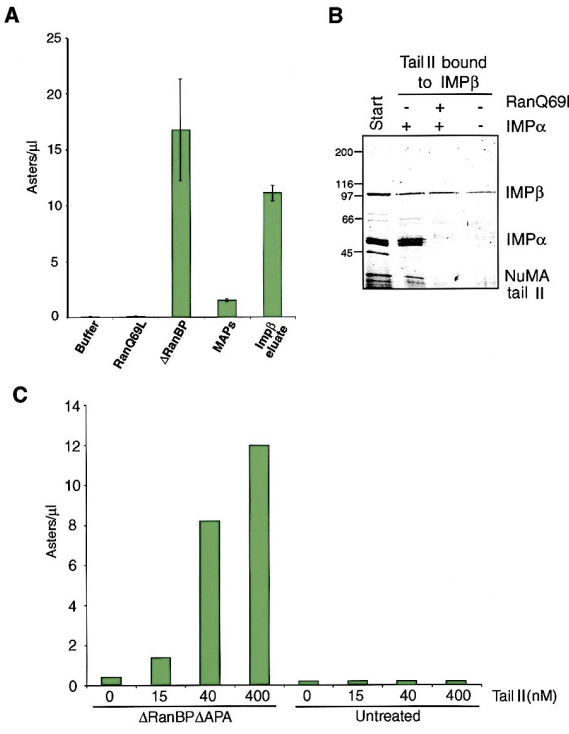

Figure 7. NuMA Is a Component of APA

(A) The addition of MAPs or importin β binding proteins can restore aster formation in the ΔRanBPΔAPA extract. ΔRanBPΔAPA extract was combined with either buffer alone, 40 μM RanQ69L, a 1:20 dilution of the ΔRanBP extract, purified MAPs, or with the eluate from the importin β matrix together with 40 μM RanQ69L (see Results and Experimental Procedures for details). Error bars represent experiment to experiment standard deviation. (B) NuMA tail II interacts with the importin α/β heterodimer. S-tagged importin β and NuMA were incubated alone or in the presence of importin α with or without RanQ69L. Final concentrations were 0.4 μM importin β, 0.6 μM *Xenopus* importin α, 0.4 μM NuMA tail II, 8 μM RanQ69L. Complexes were captured by incubation with protein S-Affiprep beads, separated by SDS-PAGE and stained with Coomassie. (C) NuMA tail II restores aster formation in the ΔRanBPΔAPA extract. Increasing amounts of NuMA tail II were added to the ΔRanBPΔAPA extract. NuMA tail II had no effect in undepleted extracts at these concentrations. Note: the endogenous NuMA concentration in *Xenopus* egg extracts is ~50 nM (Merdes et al., 1996).

division. Increasing evidence suggests that mitotic chromatin plays an important role in microtubule stabilization and organization. In this study, we have characterized and identified factors that function downstream of mitotic chromatin in spindle formation. We demonstrate that the small GTPase Ran is required to mediate the chromatin effects on microtubules. Ran depletion from *Xenopus* extracts inhibited spindle formation upon the addition of demembranated sperm nuclei or chromatin beads. Furthermore, the chromatin-associated nucleotide exchange factor RCC1 could no longer promote ectopic formation of microtubule asters in the Ran-depleted extract.

Ran does not act directly on microtubules, but regulates their organization and stabilization through an inhibitory activity that was identified here as importin β. The initial observation was that removal of RanGTP binding proteins from the extract (ΔRanBP extract) using a RanQ69L matrix induced the spontaneous formation of aster-like structures and thus mimicked the addition of RanGTP. The spontaneous microtubule polymerization could be specifically inhibited by the cargo binding domain of importin β. Importin β not only inhibited the formation of microtubule-containing structures in ΔRanBP extracts, but also prevented spindle formation in the presence of sperm nuclei or DNA beads. Furthermore, the cargo binding domain of importin β was able to inhibit spindle formation in somatic cells in vivo. Dramatic effects on microtubule structures could be observed after microinjection of importin β [71–876] into PtK1 cells and most cells failed to progress through mitosis. Since cells were microinjected during late prophase or during prometaphase, we can exclude secondary effects through a block in nucleocytoplasmic transport. Therefore, the mitotic RanGTP-importin β pathway that we have identified here seems to be conserved and functions in somatic cells.

How does importin β inhibit spindle formation? We provide evidence that importin β sequesters multiple cargoes that are required for the formation of stable microtubule structures. There are at least two distinct aster promoting activities (APAs) present in *Xenopus* extracts. One activity can be released from importin β by the addition of the IBB fragment. This activity is shown to be inhibited through importin α. A second activity functions independently of importin α and may interact directly with importin β. We identify the nuclear mitotic apparatus protein NuMA as one importin α-dependent APA component. NuMA is a microtubule-associated protein that plays an important role in spindle organization and interacts with the dynein/dynactin complex (Gaglio et al., 1995; Merdes et al., 1996). In support of a functional role for NuMA in microtubule stabilization, its carboxy-terminal tail II domain has previously been shown to have microtubule binding activity, and at high concentrations (7 μM) can induce aster formation in egg extracts (Merdes et al., 1996). At the low concentrations used here (15–400 nM), NuMA tail II did not cause aster formation in untreated extracts presumably because it is inhibited by the excess of free importin α and β (endogenous concentration of importin α and importin β are ~3 μM and ~2 μM, respectively; Görlich et al., 1994; Ribbeck et al., 1998). However, in extracts from which importin α/β had been depleted, NuMA tail II was able to spontaneously induce microtubule asters at concentrations as low as 15 nM. In addition to NuMA, several other microtubule-associated or microtubule motor proteins, including MKLP-1/CHO1, XKCM1, XCTK2, XMAP310, and TPX2 have been shown to be nuclear in interphase (Nislow et al., 1992; Walczak et al., 1996, 1997; Andersen and Karsenti, 1997; Wittmann et al., 2000). Although the nuclear import receptor has not been identified for these proteins, it is conceivable that they all bind either directly or indirectly to importin β. It will now be important to analyze their roles in RanGTP-induced aster formation.

Based on the results presented here, a pathway can be proposed that delineates how microtubule assembly and dynamics are regulated by mitotic chromatin (Figure 8). The key components of this cascade are the nucleotide exchange factor of Ran, RCC1, the small GTPase Ran, the nuclear transport factor importin β, NuMA, and other importin β cargoes that have aster promoting ac-

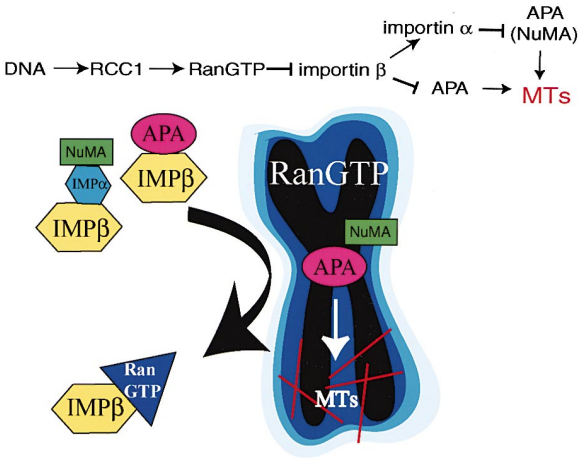

Figure 8. Schematic Model Depicting the Chromosomal RanGTP Gradient and Its Mechanism of APA Activation

The DNA-microtubule stabilization pathway starts with chromosomally localized RCC1, which generates RanGTP. RanGTP binds to the transport factor importin β, causing it to release cargoes (APAs, NuMA) that function to stabilize microtubules, thereby promoting spindle assembly (for details see text).

tivity (collectively referred to as APA). At a distance from chromosomes, spindle formation is inhibited by importin β, which sequesters and inactivates the individual APA components. High RanGAP activity in the mitotic cytoplasm ensures that Ran is predominantly kept in the GDP bound form. Ran accumulates on mitotic chromatin, presumably through its transient interaction with RCC1. RCC1 converts Ran into the GTP bound form leading to a high local concentration of RanGTP in the vicinity of chromosomes. Because importin β binds specifically to RanGTP, the individual APA components are locally discharged around chromosomes where they promote spindle formation. This effect can be mimicked by addition of high concentrations of RanGTP, removal of RanBPs, or by the competitive release of APAs from importin β. Once APAs are released from importin β, they no longer depend on RanGTP. Thus importin β and the APA components function epistatic to RanGTP in spindle assembly.

This model suggests that Ran functions during mitosis as it does in nucleocytoplasmic transport during interphase. In interphase, Ran regulates the compartment-specific delivery of import and export substrates. More specifically, RanGTP triggers the release of import cargoes from importins and promotes the binding of export cargoes to exportins in the nucleus (reviewed in Mattaj and Englmeier, 1998; Görlich and Kutay, 1999). Thus, RanGTP defines the nucleoplasm for importin β-like transport receptors and confers directionality to nucleocytoplasmic transport events (reviewed in Nachury and Weis, 1999; Görlich and Kutay, 1999). We propose that Ran functions similarly in mitosis: RanGTP activates NuMA and other APA constituents by inducing their local release from importin β exclusively in the proximity of chromatin. If Ran is removed from the extract and the RanGTP gradient is destroyed, spindle assembly is inhibited. However, if high concentrations of RanGTP are present throughout the extract, ectopic microtubule-containing figures are induced and spindle formation is no longer restricted to the proximity of chromatin. Thus, RanGTP acts throughout the cell cycle as a marker of the genome and delimits the perichromatin space, which corresponds in interphase to the nucleoplasm.

This general role of Ran is intriguing since it was recently shown that Ran is also involved in the reformation of the nuclear envelope at the end of mitosis (Hetzer et al., 2000; Zhang and Clarke, 2000). Consequently, Ran is a master regulator of several aspects of nuclear physiology. It is plausible that the role of Ran in spindle assembly preceded its function in nuclear transport or envelope assembly and that the ancestral role of Ran was to promote the localized release of a microtubule-stabilizing activity in the proximity of chromatin. After the evolution of the eukaryotic nucleus, this role may have been expanded to generally regulate transport events between the cytoplasm and the perichromatin space/nucleoplasm. It will be interesting to test if the mitotic function of Ran is conserved in unicellular organisms that do not undergo an open mitosis.

The model presented here makes a general prediction and the mitotic effects of RanGTP may not be limited to importin β. During mitosis, all nuclear import substrates should be found in a receptor-free form in the vicinity of chromatin and all nuclear export substrates should preferentially associate with their cognate receptors around chromosomes. In this context, it is intriguing that microinjection of importin β [71–876] not only inhibited spindle formation but also dramatically affected overall chromosome organization. In many microinjected cells, abberant chromatin structures were observed. This result implies that RanGTP and importin β have additional roles during mitosis. It is also conceivable that other importin β-like proteins also influence microtubule organization and possibly other mitotic events.

Experimental Procedures

Cloning, Recombinant Protein Expression, and Antibodies

The following constructs were expressed in *E. coli* as 6xHis fusion protein and purified as described: Importin β, importin β [71–876], importin β [71–601], importin β [1–601], importin β [1–380] (Chi et al., 1997), *Xenopus* importin α (Görlich et al., 1994), importin5 (Jäkel and Görlich, 1998), C-his transportin (Görlich et al., 1997), C-his CRM1 (Englmeier et al., 1999), CAS, zz-RanQ69L, Ran, RanT24N, GFP-Ran, GFP-RanQ69L (Weis et al., 1996a, 1996b; Nachury and Weis, 1999), the importin β binding domain of importin α (IBB), and importin α-ΔN65, (Weis et al., 1996a). Human RCC1 was expressed and purified as described (Klebe et al., 1993). All proteins were dialyzed against XBG Buffer (10 mM K-Hepes pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM sucrose, 5% glycerol).

The expression vectors for GFP-Ran (pKW720) and GFP-RanQ69L (pKW721) were constructed by amplifying the GFP coding sequence by PCR and inserting it between the SphI and the HindIII sites present between the 6xHis coding sequence and the Ran coding sequence of pQE-Ran and pQE-RanQ69L, respectively. Antibodies against recombinant human importin β, human Ran, human CAS, and *Xenopus* RCC1 were raised in rabbits and affinity-purified with the respective antigen. All antibodies were monospecific on blots (data not shown).

Extracts

10,000 × g cytoplasmic extracts of *Xenopus* eggs arrested by CSF were prepared as described (Murray, 1991). TexasRed-labeled tubulin prepared from calf brain (Hyman, 1991) was added to a final

concentration of 0.2 mg/ml. DNA beads and chromatin bead spindles were assembled as described (Heald et al., 1996).

For aster assays, the cytoplasmic extract was spun in a TLS-55 rotor for 15′ at 200,000 × g, the cytoplasmic layer was collected, and the extract was flash-frozen in liquid nitrogen. All reactions were performed in a 20 μl reaction volume unless indicated otherwise. Reactions were fixed and spun onto coverslips as described (Heald et al., 1997). Microtubule asters were scored as structures containing more than 10 MTs, well focused in a radiating fashion, and in which the diameter was greater than 4 μm.

Preparation of RanBP-Depleted Extract

A protein A-fusion of RanQ69L (ZZ-RanQ69L) was added to 200 μl of 200,000 × g extract at a final concentration of 25 μM. The extract was incubated for 20 min on ice and then mixed with 40 μl of IgG-Sepharose beads. After a 25 min incubation at 4°C, the beads were pelleted. The supernatant was subjected to another cycle of ZZ-RanQ69L addition and retrieval resulting in the RanBP-depleted (ΔRanBP) extract. Mock-depleted extract were treated identically but without addition of Ran.

Importin β Affinity Depletions and Interaction Assay

Proteins were coupled to Affiprep-10 beads (Biorad) at a ratio of 85 pmoles of protein per μl of matrix. The efficiency of coupling was typically 65%–75% (yielding ~60 pmol protein per μl of matrix). 200 μl of extract (10,000 × g for the depletion of Ran, 200,000 × g for all other experiments) was added to 100 μl of beads and rotated at 4°C for 30 min. Beads were pelleted and the supernatant was assayed for either aster formation, or for sperm or bead spindle assembly.

Retrieved beads were washed six times with 500 μl XB buffer and bound proteins were eluted with XBG buffer + 2 M $MgCl_2$. Proteins were precipitated with methanol/chloroform/H_2O and half of the sample was separated on a 7%–16% gradient SDS-PAGE gel.

APA Assay and Microtubule-Associated Proteins

ΔRanBP extracts were depleted by the importin β [71–876] matrix and frozen in 50 μl aliquots in liquid nitrogen. Δ90 cyclinB was added to the extract (1 μM final concentration) to ensure that the mitotic state of the extract was maintained.

To elute proteins from the importin β matrix, S^{D14N}-importin β was bound to protein S-beads and incubated with the ΔRanBP extract for 45 min at 4°C. Beads were washed with EB buffer (50 mM Hepes pH 7.6, 50 mM NaF, 80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.1 mM Na_3VO_4, 1 mM DTT, and protease inhibitors). Complexes were eluted with EB buffer containing 1 mM S-peptide and dialyzed against XBG. MAPs were prepared as described (Andersen, 1997) and desalted against XBG.

Video Microscopy and Quantitation of Fluorescence Signal

Images were recorded with either a Photometrics Sensys or a Hamamatsu C4742 cooled CCD camera mounted on a Nikon E600 microscope. The cameras were controlled by either Image Pro plus (Media Cybernetics) or Metamorph (Universal Imaging), respectively. The fluorescence intensity of sperm-associated asters was quantified using the "measure IOD" function of Image Pro plus. 30 to 50 sperm-associated asters were quantified for each condition. For real-time observations of GFP-Ran and Texas-Red tubulin signals, 2 μl of the reaction mixture was spotted onto a glass slide. All reactions were performed at 20°C.

Microinjection Experiments

Purified importin β [1–380] and importin β [71–876] were dialyzed against injection buffer (IB, 50 mM K-glutamate, 0.5 mM $MgCl_2$, pH 7) and concentrated to 650 μM and 330 μM, respectively. 5 μl of importin construct and 0.4 μl of 12 mg/ml X-rhodamine tubulin were combined and centrifuged at 77,000 × *g* for 20 min at 4°C in a S55-S rotor in a micro-ultracentrifuge (RC-M120GX, Sorvall), and then injected into PtK1 cells grown on coverslips using an Eppendorf microinjector. Between 2 and 13 prophase/prometaphase cells per coverslip were identified by phase microscopy and injected over a 20 min period, then returned to a 37°C 5% CO_2 incubator. Coverslips were processed 60 min after the start of injections by first permeabilizing in 0.5% Triton X-100 in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgSO4, pH 7), fixing in 4% formaldehyde, 0.5% glutaraldeyhyde/PHEM, and rinsing in PBS. Chromosomes were stained with SYTOX green (Molecular Probes) for 1 min diluted 1:10,000 in PBS.

Images of fixed cells were collected on a Nikon TE-300 Quantum microscope equipped with an Ultraview (Perkin Elmer) dual spinning disk real-time confocal scanner. Confocal images were collected with a Hamammatsu Orca II cooled CCD camera. Z-series of optical sections were collected of each wavelength at 1 μm intervals using a focus stepper motor (Ludl). Equipment was controlled by MetaMorph software. Images at each optical depth were color encoded and combined into an RGB overlay, then projected into a single plane using the 3-D reconstruction function in MetaMorph.

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