Phosphorylation of Maskin by Aurora-A Is Regulated by RanGTP and Importin β

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Mitotic spindle assembly in Xenopus egg extracts is regulated at least in part by importin β and its regulator, the small GTPase, Ran. RanGTP stabilizes microtubules near the chromosomes during spindle assembly by selectively releasing spindle assembly factors from inhibition by importin α/β in the vicinity of the chromosomes. Several spindle assembly factors are regulated in this manner. We identified maskin, the Xenopus member of the transforming acidic coiled coil family of proteins, as a potential candidate in a two-step affinity chromatography approach designed to uncover additional downstream targets of importin α/β in mitosis. Here, we show that although maskin lacks a canonical nuclear localization sequence, it binds importin β in a RanGTP-regulated manner. We further show that importin β inhibits the regulatory phosphorylation of maskin by Aurora-A. This suggests a novel mechanism by which importin β regulates the activity of a spindle assembly factor.

Chromosomes are segregated during cell division by the mitotic spindle, an elaborate macromolecular assembly composed of microtubules (MTs), MT motor proteins, and many non-motor MT-associated proteins (MAPs) (1). Spindle assembly is driven by the selective stabilization of MTs near the chromosomes and their organization into a bipolar array. Two pathways contribute to bipolar spindle formation in most animal cells. In one pathway, MTs are nucleated by centrosomes and are captured by kinetochores on the chromosomes (“search and capture”). In the second pathway, MTs are nucleated in the vicinity of the chromosomes and are subsequently organized into bipolar spindles.

It has become increasingly clear over the past few years that spindle assembly is regulated by proteins that, during interphase, are involved in nucleocytoplasmic trafficking (2–7). At the heart of this regulatory mechanism for mitotic spindle assembly lies importin β (8, 9). During interphase, importin β (mostly in concert with its adaptor protein, importin α) serves as the major nuclear transport receptor in the cell (10). It interacts with patches of basic amino acids (nuclear localization sequences (NLSs)) on proteins destined for the nucleus. The interactions between importin β and its “cargos” are regulated by the small GTPase, Ran (10). Binding of RanGTP (but not RanGDP) to importin β destabilizes the interaction between importin β and its cargo by causing importin β to adopt a conformation that facilitates cargo release (11). Thus, importin β binds its cargo when RanGTP is low, and releases cargo in the presence of RanGTP. By restricting the production of RanGTP to the vicinity of chromosomes, the cell uses this loading/unloading cycle to drive NLS-containing proteins into the nucleus during interphase (10, 11). A similar importin β binding/release cycle is thought to take place during mitosis in metazoan, albeit in the absence of the strict compartmentalization provided by the nuclear envelope (6, 7).

Regulating the activities of proteins that bind, stabilize, destabilize, and/or organize MTs allows rearrangement of the interphase MT array into a mitotic spindle. Several key mitotic spindle assembly proteins, including TPX2, NuMA, XCTK2, XKid, Rae1, Xnf7, HURP, and NuSAP, have been shown to bind importins during mitosis (8, 9, 12–20). Although the molecular mechanism of importin β inhibition is not fully understood, it is clear that removal of importin β is a prerequisite for the function of these proteins in mitotic spindle assembly. Importins thus serve as potent inhibitors of spindle assembly. As is true for NLS-containing proteins during interphase, release of spindle assembly factors from the inhibitory effect of importin β during mitosis requires RanGTP. Because RanGTP is found mainly in the vicinity of mitotic chromosomes (21–23), MT stabilization and spindle assembly preferentially take place near chromosomes in mitotic cells. However, a portion of RanGTP also localizes to centrosomes where it presumably regulates importin β binding to spindle assembly factors that act mainly at or near the minus ends of MTs (24, 25). Consistent with this idea, importin α and β have also been found at spindle poles during mitosis (25, 26).

Maskin is a Xenopus spindle assembly factor that stabilizes MTs and functions at centrosomes (27–29). It is a member of the highly conserved transforming acidic coiled coil (TACC) protein family (30–32). Members of this family share a ~200-amino acid C-terminal coiled coil “TACC” domain that targets the TACC proteins to centrosomes (28, 31). TACC proteins stabilize MTs (at least in part) by recruiting members of the

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3 The abbreviations used are: MT, microtubule; MAP, microtubule-associated protein; TACC, transforming acidic coiled coil; NLS, nuclear localization sequences; GST, glutathione S-transferase; PIPES, 1,4-piperazinediethane-sulfonic acid.
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XMAP215/TOG protein family to the centrosome (32, 33). Recombinant Xenopus maskin TACC domain alone is sufficient to rescue centrosome defects brought about by depleting maskin from egg extracts (27, 28), suggesting that the TACC domain plays an important role in centrosome function. The localization and activity of maskin are regulated by Aurora-A (28, 29, 34). Aurora-A is an essential protein kinase required for mitosis and centrosome maturation, a process in which centrosomes acquire additional MT nucleating material at the onset of mitosis (35–37). Phosphorylation of maskin by Aurora-A. These findings suggested that the regulation of TACC protein function by Aurora-A kinase is evolutionarily conserved.

Here, we report isolating maskin in an assay designed to identify MT-binding proteins that are regulated by importin β. We show that maskin interacts with importin β in vitro, and this interaction is regulated by RanGTP. The direct interaction between maskin and importin β was unexpected because maskin lacks a canonical NLS. Using truncation mutants to map the importin β binding domain of maskin, we found that the central portion of maskin is important for importin β binding, but efficient binding requires the full-length protein. Most importantly, we show that importin β binding inhibits the phosphorylation of maskin by Aurora-A. These findings suggest a role for importin β in regulating maskin activity at the centrosome.

EXPERIMENTAL PROCEDURES

Expression and Cloning—Fusion proteins were expressed in Escherichia coli strain C41(DE3) (42) and purified by affinity chromatography using their respective tags: Hisα-importin α (described in Ref. 8); Hisβ-importin β (also has an S-tag; Ref. 8); Hisα-RanL43E or RanT24N (described in Ref. 5); Hisα-Aurora-A (43); GST-maskin (27) and GST-maskin truncation mutants (see below). All proteins were dialyzed against XB (10 mM K-HEPES, pH 7.6, 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose) and stored at −80 °C in small aliquots.

Maskin truncations were generated using the following primer pairs (the cloning vector for each construct is indicated in parentheses, restriction sites introduced by the primer are underlined and are identified by name following the sequence, and stop codons are indicated in bold): 1–774 (pGEX4-T2rTEV; see Ref. 27); 5’-CGTTGAAATCGACTATCTAGAG (XbaI), 3’-GTCAGCTCTTAGCTCTTAACTTCACAATCTGTC (SacI); the fragment amplified with these primers was used to replace the XbaI/Sacl fragment of full-length maskin (27). 364–500 (pGEX6-P2); 5’-CGCTAAACCTTGGTGCTGCT (EcoRI), 3’-GTCAGCTCTTAGCTCTTAACTTCACAATCTGTC (SacI); the fragment amplified with these primers was used to replace the XbaI/Sacl fragment of full-length maskin (27). 364–500 (pGEX6-P2); 5’-CGCTAAACCTTGGTGCTGCT (EcoRI), 3’-GTCAGCTCTTAGCTCTTAACTTCACAATCTGTC (SacI); the fragment amplified with these primers was used to replace the XbaI/Sacl fragment of full-length maskin (27).

Isolation of Mitotic MAPs—Mitotic MAPs were prepared as described (47) with the following modifications. Concentrated mitotic extracts (5 ml/purification) were diluted with 1 volume of buffer (50 mM sucrose, 10 mM K-HEPES (pH 7.7), 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride). This mixture was centrifuged at 192,000 × g for 40 min at 4 °C. The reaction was then moved to 22–25 °C and incubated for 15 min. The volume of protein solution added never exceeded 20% of total extract volume. Images were taken with a Photometrics CoolSnap HQ cooled CCD camera (Roper Scientific, Inc.) through a ×60/1.4 NA plan apochromatic objective mounted on a Nikon Eclipse E800 fluorescence microscope. Images were obtained using MetaMorph software and processed using Adobe Photoshop.

Antibodies—Antibodies against recombinant full-length maskin were described in Ref. 27 and for immunofluorescence were directly labeled with Oregon Green 488 maleimide according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). TPX2 antibodies were described in Ref. 43. Importin α antibodies were a kind gift from M. Dasso (National Institutes of Health). GST antibodies were a kind gift from S. Bednarek (University of Wisconsin, Madison, WI). Control preimmune IgG was from the serum of rabbits before inoculation with maskin. Importin β antibodies were purchased from Transduction Laboratories (Lexington, KY). Secondary antibodies used for Western blotting were purchased from Sigma.

Xenopus Egg Extract Preparation and Light Microscopy—Cytostatic factor-arrested (44, 45) or interphase (46) extracts were prepared from Xenopus eggs as described. Maskin (0–40 μM) and/or importin β (as indicated in Fig. 4) were added to mitotic extract on ice; the reaction was then moved to 22–25 °C and incubated for 15 min. The volume of protein solution added never exceeded 20% of total extract volume. Images were taken with a Photometrics CoolSnap HQ cooled CCD camera (Roper Scientific, Inc.) through a ×60/1.4 NA plan apochromatic objective mounted on a Nikon Eclipse E800 fluorescence microscope. Images were obtained using MetaMorph software and processed using Adobe Photoshop.

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β, S-protein beads were added to the mixture, which was incubated for an additional 60 min. The beads were collected by a brief spin, and washed three times with XB and once with BRB80. Bound proteins were eluted by boiling the beads in SDS sample buffer. Eluted proteins were separated by SDS-PAGE and visualized by Coomassie staining or Western blotting.

**Pull-out Assays**—Purified recombinant proteins were incubated together or with mitotic egg extract (as indicated in Figs. 2 and 5) for 30 min at 4 °C. Glutathione-agarose was added to retrieve GST-maskin, or S-protein-agarose was added to retrieve importin β. For the experiment shown in Fig. 5B, GST-tagged maskin truncation mutants were first covalently linked to Dynabeads M-270 Carboxylic Acid (Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions, and then incubated in mitotic egg extracts. The beads and associated proteins were collected by a brief spin and washed 3 times with XB and once with BRB80. Bound proteins were eluted by boiling the beads in SDS sample buffer. Eluted proteins were separated by SDS-PAGE and visualized by Coomassie staining or Western blotting.

**Immunoprecipitations**—To immunoprecipitate maskin, anti-maskin antibodies were conjugated to Affi-prep protein A beads (Bio-Rad) as described (48). Antibody-coupled beads (50 μl) were added to the extract and incubated for 1 h at 4 °C. Beads were collected by brief centrifugation, the immunoprecipitated were washed with H100, and analyzed by SDS-PAGE and Coomassie staining, or were transferred to nitrocellulose membranes. The equilibrium dissociation constant was calculated using GraphPad Prism Software.

**Kinase Assays**—Kinase assays were performed as described (28, 29), with the following modifications: maskin (0.6 μM), importin β (3–12 μM), importin α (0.6 μM), and RanL43E (24 μM) were incubated in kinase buffer for 30 min at 4 °C. Aurora-A (0.06 μM) and [γ-32P]ATP (8 μM; Redivue, 3,000 Ci/mmol; obtained from Amersham Biosciences) were added and the mixture was incubated at 22–25 °C for 15 min. Reactions were stopped by addition of SDS sample buffer and separated by SDS-PAGE. Incorporation of label was detected by autoradiography using a Phospholimager and was quantitated using Adobe Photoshop software.

## RESULTS

**The Xenopus Spindle Assembly Factor Maskin Is a Potential Importin β Target**—Several proteins that function to regulate mitotic spindle assembly or MT bundling have been shown to interact with importin β in mitosis (7, 49). To identify additional potential targets for regulation by importin β, we reasoned that at least a subset of proteins that regulate mitotic spindles through importin β should directly bind to both MTs and importin β. Other potential importin β binding targets, which were not selected for in our assay, might be regulatory factors that interact with MTs transiently, or not at all. To identify candidate MT binding targets, we isolated mitotic MAPs from Xenopus egg extracts (see “Experimental Procedures”), supplemented them with importin α (which in some cases acts as an adaptor between NLS-containing proteins and importin β), and loaded these onto an importin β affinity chromatography column. A distinct subset of mitotic MAPs specifically interacted with importin β, either directly or in the presence of importin α (marked with asterisks in Fig. 1A). These proteins were excised from the gel, digested with trypsin to generate peptides, and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry to analyze peptide masses. Data base analysis of the peptide masses revealed that 11 peptides of the ~100-kDa protein matched the expected masses of tryptic peptides of TPX2, covering 17% of the TPX2 sequence (not shown). Western blotting confirmed the presence of TPX2 in our final fraction (Fig. 1B). This result is consistent with previous reports that TPX2 is a downstream target of the Ran pathway in mitotic spindle assembly (12, 43).

Matrix-assisted laser desorption ionization time-of-flight analysis further identified the ~150-kDa protein as the Xenopus TACC protein, maskin, and this result was confirmed by Western blotting (Fig. 1B; 12 peptides matched). Maskin is involved in centrosome and spindle assembly in Xenopus egg extracts (27–29). Interestingly, sequence analysis revealed that maskin lacks a canonical importin β binding domain. Maskin binding to importin β appeared to be independent of importin α, but more efficient in its presence (Fig. 1B; see also Fig. 2). We concluded that despite the absence of an NLS, maskin is a potential importin β target during mitosis.

**Maskin Interacts with Importin β in Vitro**—Two possibilities could explain why the non-NLS-containing maskin was identified in an assay designed to uncover proteins that are regulated by importin β: 1) maskin interacts with importin β via an unconventional importin β binding domain; or 2) maskin interacts with importin β indirectly via other, NLS-bearing proteins. To distinguish between these possibilities, we tested the ability of maskin to interact with importin α and/or β in vitro, in the absence of the other MAPs. For this, recombinant proteins were incubated in vitro. Complexes were selectively isolated by affinity chromatography using the unique tag on one of the proteins (“pull-out” experiments). In one set of pull-out experiments, we incubated protein S-tagged importin β with importin α and/or maskin, isolated importin β using its protein-S tag, and probed for bound maskin (Fig. 2A). These experiments showed that maskin co-purified with importin β, and this binding was independent of importin α. Moreover, the interaction between importin β and maskin was abolished in the presence of RanL43E, a Ran point mutant that mimics its GTP-bound state (Fig. 2A). In another set of experiments, we incubated GST-tagged maskin with importin α and/or importin β, re-isolated GST-maskin using glutathione beads, and probed for bound importins (Fig. 2B). We found that maskin interacted directly with importin β as well as importin α, and the interaction between maskin and importin β was enhanced by the presence of importin α. Importantly, the maskin/importin β interaction was abolished by the addition of RanL43E but not by RanT24N (a RanGDP mimic) (Fig. 2B), suggesting that the in vitro interaction between maskin and importin β is regulated by RanGTP.

The interaction between importin β and maskin was saturable: pull-out experiments with GST-maskin and increasing amounts of importin β (in the presence of constant levels of...
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FIGURE 1. Several MAPs bind specifically to importin β. Mitotic MAPs isolated from Xenopus egg extract were incubated with importin β and/or importin α. S-protein beads were added to retrieve importin β and associated proteins (affinity chromatography). One set of controls (as indicated) contained no importin β and thus measures nonspecific binding to S-protein beads. A, Coomassie-stained gel of importin-β affinity chromatography. Lanes 1–3, column input: MAPs (lane 1), recombinant importin α (lane 2), recombinant importin β (lane 3); lane 4, eluate from the importin β affinity column; MAPs that specifically interact with the importin β column are indicated by asterisks; lane 5, eluate from control column (no importin β); mitotic MAPs incubated with importin α and β eluted from an S-protein column; lane 6, flow-through of MAPs that did not bind to the importin β affinity column (matches with lane 4); lane 7, flow-through of MAPs that did not bind to control S-protein column (matches with lane 5); lane 8, molecular mass markers; size in kDa, indicated on the right. B, Western blots to detect maskin (top panel) or TPX2 (bottom panel) from importin-β affinity chromatography. Lane 1, S-protein beads incubated with recombinant importin α (control); lane 2, S-protein beads incubated with recombinant importin β, without MAPs (control); lane 3, mitotic MAPs eluted from the importin β affinity column in the presence of importin α; lane 4, eluted mitotic MAPs incubated with importin β only (no importin α); lane 5, mitotic MAPs incubated with importin α only; lane 6, extract control.

importin α) showed that the amount of importin β that co-purified with maskin reached a maximum that was not increased by an excess of importin β (Fig. 2C). Nonlinear regression analysis determined that the apparent equilibrium dissociation constant (Kd) for the interaction between maskin and importin β was 1.3 ± 0.2 µM (Fig. 2D).

Maskin Interacts with Importin β in Xenopus Egg Extracts—The result that maskin binds to importin β in vitro supported the idea that maskin might possess an unconventional importin β binding domain. We used four criteria to test this hypothesis: 1) we examined a potential maskin/importin β interaction in egg extracts by co-immunoprecipitation; 2) we examined the subcellular localization of maskin in interphase extracts; 3) we examined the effect of adding exogenous maskin into mitotic extracts; and 4) we mapped the importin β binding region of maskin.

To determine whether maskin and importin β interacted in extracts, we immunoprecipitated maskin from mitotic Xenopus egg extracts and probed for the presence of importin β (Fig. 3A). We found that importin β co-immunoprecipitated with maskin, suggesting that importin β binds maskin in mitotic extracts (see also GST pull-downs in Fig. 5). Importantly, addition of RanL43E, but not RanT24N, to the extract before immunoprecipitation weakened the maskin/importin β interaction but had no effect on the interaction between maskin and XMAP215 or Aurora-A (Fig. 3A).

Further support for the notion that maskin and importin β interact came from experiments using interphase Xenopus egg extracts. These extracts readily assemble nuclei around exogenously added sperm chromatin (50). If maskin interacts with importin β in the extracts, we expect maskin to accumulate in nuclei. Nuclei assembled in egg extracts were probed for maskin using directly labeled affinity purified anti-maskin antibodies (Fig. 3B). This analysis showed that maskin accumulated in nuclei assembled in vitro. There are two possibilities to explain this result: 1) maskin was imported into the nucleus, presumably in an importin β dependent pathway; or 2) maskin was brought into the nucleus with the sperm chromatin. To address this point, we both stained demembranated sperm chromatin with anti-maskin antibodies (Fig. 3C) and assembled nuclei in maskin-depleted egg extracts (not shown). Immunofluorescence analysis showed that sperm chromatin was mostly devoid of maskin staining (Fig. 3C). Similarly, only a very weak maskin signal was detected in nuclei assembled in extracts mostly depleted (~70%) of maskin (not shown). We concluded that maskin was imported into nuclei in interphase Xenopus egg extracts. Because importin β is the major nuclear import receptor in vertebrate cells (7, 10), this strongly suggests that maskin interacted with importin β.

Maskin Addition to Xenopus Egg Extracts induces Aster and Spindle Formation—As another approach to test whether maskin is downstream of RanGTP and importin β, we tested the effect of adding high concentrations of recombinant maskin to mitotic egg extracts. We reasoned that if maskin was able to...
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RanL43E addition resulted in formation of asters and spindles (Fig. 4, A and B). Significantly, addition of 10 μM bacterially expressed maskin to mitotic extracts also resulted in the formation of asters and spindle-like structures (Fig. 4, A and B), although at 10-fold reduced efficiency. Increasing the amount of maskin added did not increase the number of structures formed, but instead exerted a dominant negative effect: the asters were unfocused at intermediate maskin concentrations (20 μM), and were disrupted at high maskin concentrations (40 μM) (Fig. 4A). Addition of importin β to the reaction was able to overcome the maskin-induced MT assembly in a dose-dependent manner (Fig. 4C), suggesting that the effect of maskin on MT assembly was mediated by importin β. We concluded that exogenous maskin induced MT assembly by competing with spindle assembly factors for binding to importin β, supporting the notion that maskin interacts with importin β in mitosis.

The Importin β Binding Domain of Maskin Maps to Amino Acids 501–636—Next, we sought to map the importin β binding region of maskin. For this, we generated a series of truncation mutants of maskin (as N-terminal GST fusion proteins to aid in purification and detection; Fig. 5A). The truncations were assayed for importin β binding using two approaches: 1) truncations were incubated in Xenopus egg extract, re-isolated using glutathione-agarose beads, and the amount of importin β present in the pull-outs was quantitated by Western blotting (Fig. 5B); or 2) the truncations were added to Xenopus egg extracts (20 μM final concentration) supplemented with rhodamine tubulin, and their activity was scored as the number of asters formed in 50 fields. The results of these assays (summarized in Fig. 5) showed that whereas some truncation mutants bound importin β more efficiently or induced more asters than others, none of the fragments did so as well as the full-length protein. The truncation mutants behaved similarly in both assays, i.e., those mutants that bound more importin β (e.g., the fragment encompassing amino acids 364–774 or 501–636) also induced more asters. A fragment derived from the central portion of maskin (amino acids 501–636) appeared to be the smallest fragment that showed activity in both assays (Fig. 5). These results suggested that the importin β binding domain of maskin most likely includes residues in the central portion of maskin, but efficient binding of importin β might be dependent on a particular three-dimensional conformation.

FIGURE 2. Maskin binds to importin β in vitro, and this interaction is regulated by RanGTP. Purified recombinant components were combined and incubated in vitro, and bound proteins were retrieved using a specific tag on one of the components, as indicated. A, proteins that co-purify with S-protein-tagged importin β were separated by SDS-PAGE, Western blotted, and probed for maskin (top panel), importin β (middle panel), or importin α (bottom panel). Binding proteins were analyzed by Western blotting rather than Coomassie staining to eliminate background. Lane 1, maskin and importin α incubated with S-protein beads; lane 2, maskin and importin β; lane 3, maskin, importin α, and importin β; lane 4, maskin, importin α, and importin β, and RanL43E; lane 5, RanGTP mimic; lane 6, maskin, importin α, and RanL43E; lane 7, maskin incubated with S-protein beads. Lanes are from left to right. B: panel a, Western blots of proteins that co-purify with GST-maskin were probed for maskin (upper panel) or importin β (bottom panel). Lane 1, maskin and importin β; lane 2, maskin and importin α; lane 3, maskin, importin α, and importin β; lane 4, maskin, importin α, and importin β, and RanL43E; lane 5, RanGTP mimic; lane 6, maskin, importin α, and RanL43E; lane 7, maskin incubated with S-protein beads. Lanes are from left to right. Panel b, quantitation of the amount of importin β that co-purifies with maskin under the conditions described in a. The presence of RanL43E, but not RanT24N, prevents importin β from co-purifying with maskin. **, p < 0.001 (standard normal distribution). The graph represents the average of 3 independent experiments ± S.D. C, importin β binding to GST-maskin is saturable. GST-maskin (3 μM) was incubated with increasing amounts of importin β. All reactions also contained 0.1 μM importin α. Proteins were retrieved on GST beads, and the amount of importin β (upper panel) that co-purifies with GST-maskin (lower panel) was detected by Western blotting. The concentration of importin β input was as follows: lanes 2, 0.3 μM; lane 3, 0.6 μM; lane 4, 0.8 μM; lane 5, 1.2 μM; lane 6, 1.4 μM; lane 7, 1.7 μM; lane 8, 2.0 μM; lane 9, 2.8 μM; lane 10, 3.4 μM; lane 11, 5.0 μM. D, quantitation of the amount of importin β that co-purifies with GST-maskin under the conditions described in C. The graph represents four independent experiments (± S.E.) and was generated using GraphPad Prism software.

FIGURE 3. Maskin binds importin β in the egg extract and localizes to the nucleus. A, maskin immunoprecipitation in the presence of RanT24N (lane 1) or RanL43E (lane 2). Western blot probed for XMAP215, Aurora-A, importin-β, and maskin, as indicated on the left. B, nuclei assembled in interphase extract by addition of sperm chromatin were stained with affinity purified anti-maskin antibody (b) or preimmune serum (d); the corresponding DNA stain is shown in a and c, respectively. Scale bar, 10 μm. C, maskin staining of demembranated sperm chromatin incubated in Xenopus egg extract in the presence of nocodazole (a and b) or fixed and imaged directly (without incubation in egg extract) (c and d). DNA stain (a and c) or maskin immunofluorescence (b and d). Arrowheads in b and d point to basal bodies. Scale bars, 10 μm.

interact with importin β in mitotic egg extracts, it might be able to induce MT assembly by liberating spindle assembly factors from the inhibitory effect of importin β by competing for importin β binding, as was previously reported for the importin β-binding protein of NuMA (8), and the artificial importin β-binding protein, BSA-NLS (12). As expected, no MT structures formed in mitotic extracts treated with buffer alone, and
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![FIGURE 4. Addition of maskin to egg extracts induces asters and spindle-like structures. A, micrographs of MT structures formed within 15 min in egg extract treated with 25 μM RanL43E (a and b) or 10 μM (c and d), 20 μM (e and f), or 40 μM (g and h) maskin. Scale bar, 25 μm. B, micrographs of spindle-like structures formed after 90 min of incubation in extract treated with 25 μM RanL43E (a and b) or 10 μM maskin (c and d). Scale bar, 25 μm. C, masking-induced aster formation is inhibited by exogenously added importin β. Importin β and maskin were added to egg extracts at the beginning of a 15-min incubation. The graph shows the average numbers of asters formed in three independent experiments (± S.D.), normalized against the "no importin β" control.](image)

**DISCUSSION**

Mitotic spindle assembly requires the precise spatial and temporal regulation of proteins involved in modulating MT nucleation and dynamics. The Ran pathway plays an essential role in this process. Considering the compositional and functional complexity of the mitotic spindle, it is reasonable to expect that the activities of many proteins are modulated by Ran and its downstream effectors. This is underscored by the observation that Ran stabilizes MTs in *Xenopus* egg extracts by increasing their rate of “rescue” (51, 52), yet none of the eight Ran-regulated proteins identified to date affect rescue frequency. It is also interesting to note that with the exception of HURP (18, 19), all Ran-regulated spindle assembly factors localize to the spindle poles during mitosis. Paradoxically, the spindle poles are the part of the spindle that is farthest away from the chromosomes, where the RanGTP concentration is highest. Thus, it is clear that important components of this emerging picture are still missing.

We set out to isolate additional downstream targets of Ran, and designed a two-step affinity purification scheme to identify potential candidates. To our surprise, our assay identified maskin as a candidate. Maskin is a centrosomal protein whose phosphorylation by the Aurora-A kinase was recently shown to be essential for its activity (28, 29). Here, we describe the characterization of maskin as a novel target of Ran-regulated spindle assembly. Using five criteria we show that maskin and importin β interact directly and specifically: 1) recombinant maskin and importin β interact directly in vitro, and this interaction can be reversed by RanGTP; 2) maskin localizes to the nucleus in interphase extracts; 3) maskin addition to mitotic *Xenopus* egg extracts induces aster formation; 4) the importin β binding domain of maskin maps to its central portion; and 5) importin β inhibits the phosphorylation of maskin by Aurora-A in a RanGTP-reversible manner.

*The Importin β Binding Domain of Maskin—*Importin β interacts with a wide variety of cargos, usually by binding to short stretches of basic amino acids, or, in some cases, two basic patches that are separated by 10–12 amino acids (53). Import signals that arise by bringing together basic patches from different regions of the molecule have also been described (53).

Maskin is a highly acidic protein with an overall pl of ~4.5. Not surprisingly, clusters of basic amino acids that could serve as “classical” or “bipartite” NLSs are very rare in maskin (Fig. 5A). Three such “clusters” are found between amino acids 401 and 426 (Fig. 5B). Consistent with our observation that maskin binds to importin α, phosphorylation of maskin was also inhibited by importin α, but to a lesser extent than importin β (Fig. 6C). Inhibition by importins was partially reversed by the addition of RanL43E, but not RanT24N, to the reaction, suggesting that phosphorylation of maskin by Aurora-A is regulated by RanGTP.
Maskin Phosphorylation Is Regulated by RanGTP

FIGURE 5. The importin β binding domain of maskin maps to the central portion of maskin. A: a schematic representation of the maskin truncation mutants used in this study. The amino acids spanned by each fragment are indicated on the left. The table on the right represents a qualitative assessment of the ability of each fragment to interact with importin β in the egg extracts (GST pull-out) or to induce aster formation. b, charge distribution analysis of maskin. The average charge in the window of 5 amino acids is shown in the top trace. Positions of positive and negative charge clusters are shown in the bottom two traces, respectively. The graph was generated with the Protean module of the DNAstar software. B, GST maskin truncations were incubated in Xenopus egg extract and re-isolated using glutathione-agarose beads. a, quantitation of the amount of importin β that co-purifies with maskin truncation mutants. The graph represents an average of four independent experiments ± S.E.; *, p ≤ 0.05; **, p ≤ 0.01 (Student’s t test). b, importin β Western blot of a typical pull-out experiment. C, quantitation (average of three independent experiments ± S.D.) of the number of asters formed upon addition of 20 μM maskin truncation mutants (as indicated) to Xenopus egg extract, normalized to the number of asters formed in reactions containing full-length maskin.

composed of several highly negatively charged regions in its non-TACC portion, whereas the TACC domain is positively charged (the pI of the TACC domain is ~8.7), we imagine that maskin perhaps folds back on itself. This idea is supported by the observation that the TACC domain of maskin can localize to the centrosome on its own, but full-length maskin-3A (a mutant in which three important serine residues have been changed to alanines; Ref. 38) fails to accumulate at centrosomes even though the TACC domain is still present. This indicates that the non-TACC portion of maskin can obscure the centrosome targeting signal in the TACC domain. This hypothesis is further supported by the observation that the three human TACC proteins (hTACC1–3) have distinct subcellular localizations despite closely related TACC domains (54).

Maskin binds importin β directly, although the interaction is more efficient in the presence of importin α (Fig. 2). Thus, maskin joins HURP and Rae1 as a third Ran-regulated spindle assembly factor that interacts with importin β directly (16, 19). Both HURP and Rae1 shuttle through the nucleus in interphase (19, 55), and we wondered if maskin might also shuttle. We found that maskin accumulated in nuclei assembled in the Xenopus egg extract in vitro. However, we have been unable to find evidence in tissue culture cells that maskin shuttles through the nucleus. It is possible that maskin behaves differently in embryonic systems and somatic cells. However, it is also possible that maskin does shuttle but its lower concentration makes it undetectable once it is dispersed over the nuclear volume. Consistent with this, maskin is ~100-fold less abundant in tissue culture cells than in Xenopus egg extracts. A third possibility is that maskin is actively retained in the cytoplasm in tissue culture cells, as has previously been reported for Xnf7 (17).

Maskin is unlikely to be unique among TACC proteins in its ability to interact with importin β. For example, all three human TACC proteins show nuclear staining in HeLa cells during interphase, and hTACC3 localizes strongly to the nucleus (54). It will be interesting to know whether human TACC proteins also interact with importin β.

Ran and the Centrosome—Although its involvement in spindle assembly was initially discovered in studies of the centrosome-independent pathway of spindle assembly, several lines of evidence are beginning to support a role for the Ran pathway in the regulation of centrosome function. For example, RanGTP associates with centrosomes throughout the cell cycle, and dissociation of AKAP450, the protein that anchors Ran, from the centrosome results in defects in MT nucleation and anchoring (24). The Ran-binding protein, RanBP1, localizes to centrosomes, and its disruption causes centrosome splitting and multipolar spindles (56–58). Furthermore, importin β (as well as importin α) localizes to spindle poles in mitosis in a TPX2- and dynemin/dynactin-dependent manner, and its overexpression causes spindle pole fragmentation (25). Evidence for a connection between the centrosome and the Ran pathway also comes from the observation that several centrosomal proteins cycle through the nucleus, including pericentrin, RanBP1, and centrin-1 (but not nenie, γ-tubulin, or AKAP450; Ref. 24). Last, Aurora-A kinase local-
Maskin Phosphorylation Is Regulated by RanGTP

FIGURE 6. Importin β inhibits the phosphorylation of maskin, and this inhibition can be overcome by RanGTP. A, schematic representation of the Aurora-A phosphorylation sites in maskin. Aurora-A phosphorylates maskin at serines 33, 620, and 626. The position of fragment 501–636 is indicated. B, importin β inhibits Aurora-A-induced phosphorylation of maskin in a dose-dependent manner. a, importin β was added at the indicated concentrations to an in vitro kinase reaction containing 0.6 μM maskin and 0.06 μM Aurora-A. Upper panel, autoradiograph; lower panel, Coomassie-stained gel showing the region of the gel that contains maskin. b, quantitation of the relative amount of maskin phosphorylation. The graph represents three independent experiments; error bars, S.E. C, the importin β inhibition of maskin phosphorylation is overcome by RanL43E but not RanT24N. Recombinant proteins were incubated with [32P]ATP in vitro. The proteins present in each reaction are indicated above the lanes. Coomassie-stained gel. The region of the gel that contains maskin is indicated. 1. *p ≤ 0.05; **p ≤ 0.01 (Student’s t test).

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