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Aurora A kinase modulates actin cytoskeleton through phosphorylation of Cofilin: Implication in the mitotic process

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ABSTRACT

Aurora A kinase regulates early mitotic events through phosphorylation and activation of a variety of proteins. Specifically, Aur-A is involved in centrosomal separation and formation of mitotic spindles in early prophase. The effect of Aur-A on mitotic spindles is mediated by the modulation of microtubule dynamics and association with microtubule binding proteins. In this study we show that Aur-A exerts its effects on spindle organization through the regulation of the actin cytoskeleton. Aurora A phosphorylates Cofilin at multiple sites including S^3 resulting in the inactivation of its actin depolymerizing function. Aur-A interacts with Cofilin in early mitotic phases and regulates its phosphorylation status. Cofilin phosphorylation follows a dynamic pattern during the progression of prophase to metaphase. Inhibition of Aur-A activity induced a delay in the progression of prophase to metaphase. Aur-A inhibitor also disturbed the pattern of Cofilin phosphorylation, which correlated with the mitotic delay. Our results establish a novel function of Aur-A in the regulation of actin cytoskeleton reorganization, through Cofilin phosphorylation during early mitotic stages.

1. Introduction

Aurora A (Aur-A) is a member of the family of Aurora serine/threonine kinases, which play important roles in the mitotic process. Expression of Aur-A is significantly increased during late G2 when it is targeted to the centrosomes. Aur-A is responsible for centrosomal maturation and separation by recruiting α-tubulin, centrosomin, NDEL1, TACC, and LATS2 to the centrosomes [1–4]. Aur-A also regulates mitotic spindle assembly through interactions with LIMK1, TPX2, Eg5, Hurp, and XMAP215 [5–8]. Although the function of Aur-A is essential during early prophase, spindle pole localization of Aur-A is sustained through the mitotic phases, suggesting its involvement in later mitotic events. Recent studies showed a cooperative function of Aur-A and Aur-B on anaphase microtubule dynamics [9]. Aur-A expression is tightly regulated and altered expression of Aur-A results in mitotic spindle defects. Inhibition of Aur-A expression resulted in chromosome misalignment and multinucleated cells [10], whereas overexpression of Aur-A induced generation of supernumerary centrosomes, multipolar spindles, and aneuploidy. Importantly, overexpression of Aur-A is seen a variety of cancers including, breast, ovarian and prostate [11,12], which may lead to the development of aneuploidy in the cancerous cells.

In addition to its regulation of microtubule dynamics and chromosome segregation during mitosis, Aur-A has been implicated in the regulation of actin cytoskeleton. Activation of *Drosophila* Aur-A has been suggested to play a role in actin dependent asymmetric protein localization during mitosis [13]. Overexpression of Aur-A was shown to induce the up-regulation of SSH-1 leading to dephosphorylation and activation of the actin depolymerizing protein, Cofilin [14]. Aur-A also interacts with LIMK1 and Ajuba, proteins that are involved in the reorganization of the actin cytoskeleton [8,15]. Recent studies showed an indirect relationship between Aur-A and regulation of actin-dependent processes through phosphorylation of Rho kinases in *Drosophila* [16]. Nonetheless, the role of Aur-A regulation of the actin cytoskeleton has not been clearly defined.

Although not widely studied, actin has an important function throughout mitosis. During G2 phase, the actin cytoskeleton is involved in centrosome separation [17,18]. Cortical actin plays a role in the anchoring and orientation of the mitotic spindle [19,20]. Additionally, the regulation of actin dynamics is essential for the completion of cytokinesis through the formation of the contractile ring [21,22]. The dynamics of the actin cytoskeleton is regulated by the actin depolymerizing protein, Cofilin. Kinases, such as LIMK1/2 and TESK1/2, regulate Cofilin activity through phosphorylation, which prevents its binding to actin [23–27]. However, functionally active Cofilin is essential for the completion of cytokinesis. Also, LIMK1 mediated inactivating phosphorylation of Cofilin during mitosis is necessary for proper mitotic spindle orientation [28], however, the exact function of Cofilin during mitosis has yet to be determined. In this study, we

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identified Cofilin as a novel substrate of Aur-A. Aur-A regulates Cofilin activity through phosphorylation, thereby regulating actin polymerization. Additionally, we found that Aur-A is involved in the regulation of Cofilin phosphorylation during mitosis.

2. Materials and methods

2.1. Cell culture, G2/M phase enrichment, and transfection

PC-3 prostate cancer cells (ATCC) were cultured in F12 HAM containing 10% fetal bovine serum and 1% antibiotic/antimycotic. M12 prostate cancer cells (a gift from Jay Ware) were cultured in RPMI containing 5% fetal bovine serum, 10 ng/mL EGF, ITS mix (5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/ml selenium), 50 μg/mL gentamycin, and 0.1 μM dexamethasone. NIH-3T3 cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum and 1% antibiotic/antimycotic. MCF7 cells obtained as a gift from James Turkson Univ. of Hawaii were cultured in DMEM containing 10% fetal bovine serum and 1% antibiotic/antimycotic. Cells were enriched at G2/M phase as follows: Cells were synchronized at the G2/M boundary by treatment with nocodazole (3T3 600 ng/ml, 16 h; M12 80 ng/ml, 24 h). Synchronized cells were isolated by mitotic shake off and released into mitotic phases using fresh complete media. Cells were harvested at 0, 30, and 60 min after release. For experiments with Aur-A inhibitor, M12 cells were treated with MLN8237 (100 nM), BMS-5 (5 μM), both BMS-5 (5 μM) and MLN8237 (100 nM), or DMSO for 24 h, then nocodazole was added for an additional 24 h. Cells were collected by mitotic shake off and were released into mitosis with fresh media containing MLN8237 (100 nM), BMS-5 (5 μM), BMS-5 (5 μM) and MLN8237 (100 nM), or DMSO and harvested at specific time points. For ectopic expression of Cofilin, M12 cells were transfected with Cofilin-RFP using X-tremeGENE HP (Roche) and used between 48 and 72 h.

2.2. Inhibitors and antibodies

Specific inhibitors and reagents used were BMS-5 (Synkinase), DMSO (Sigma), nocodazole (Sigma), MLN8237 (a gift from Selleck), and VX-680 (a gift from Selleck). The primary and secondary antibodies used were mouse anti-human-Aur-A (Sigma), rabbit anti-human-Cofilin (Novus), rabbit anti-human-Cofilin (Pierce), rabbit anti-human-pS^3-Cofilin (Cell Signaling), mouse anti-human-GAPDH (Sigma), AlexaFluor-488 Phalloidin (Molecular Probes), rabbit anti-human-pT^{505}/T^{508}-LIMK1/2 (Cell Signaling), mouse anti-human-α-tubulin (Sigma), rabbit anti-human-SSH1 (Cell Signaling), horseradish peroxidase (HRP) conjugated goat anti-rabbit (Jackson Laboratories), HRP conjugated goat anti-mouse (Jackson, Laboratories), anti-mouse AlexaFluor-488 (Invitrogen) and anti-rabbit Cy3 (Jackson Laboratories).

2.3. Immunoblotting and Immunoprecipitation

Cells were harvested by trypsinization and lysed with RIPA lysis buffer. For immunoblots, 50 μg whole cell extracts were separated by SDS-PAGE and proteins were transferred to a PVDF membrane. Specific polypeptides were detected by reacting with the appropriate primary and secondary antibodies and protein bands were visualized using a substrate from an Immun-Star WesternC Kit (Biorad). For immunoprecipitates, 300 μg–500 μg whole cell extract was pre-cleared with Sepharose A/G beads (Santa Cruz) overnight at 4 °C. Aur-A or Cofilin was precipitated from the extract with 1.5 μg–2 μg Aur-A or Cofilin antibody at 4 °C for 6 h. Mouse IgG or rabbit IgG was used as a control. Protein–antibody complexes were pulled down with Sepharose A/G beads at 4 °C overnight. Unbound protein was removed by washing with RIPA buffer. Bound proteins were separated by SDS-PAGE and Cofilin was detected by immunoblotting using anti-Cofilin antibodies.

2.4. Recombinant protein production and purification

The coding sequence of *Cofilin* was cloned into the pET-30 vector as previously described [29], and in pCMV6-AC-RFP vector (Origene) to generate His- and RFP-tagged Cofilin. Cofilin mutants (Cofilin^{S3A}, Cofilin$^{S3A/S8A/T25A}$ and Cofilin^{S3EE}) were produced using the QuickChange XL site-directed mutagenesis kit (Agilent). The truncated Cofilin construct Cofilin$^{90–166}$ was produced by PCR amplification of the DNA fragment containing bases 268–501 of the *Cofilin* ORF and cloning into the pET-30 vector. The wild-type Aur-A and inactive Aur-A^{K162M} mutant were cloned into the pET-30 vector as previously described [8]. Recombinant Cofilin and Aur-A were expressed and purified as previously described [8,29].

2.5. Kinase assays

For *in vitro* kinase assays, purified recombinant His-tagged proteins were incubated in kinase assay buffer (50 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 10 mM EGTA, 4 mM EDTA, 50 mM $MgCl_2$, 0.5 mM DTT, 250 μM ATP) and 5 nM γ-^{32}P-ATP for 30 min at room temperature. The reaction was stopped by the addition of Laemmli sample buffer and proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue dye. Phosphorylation was detected by autoradiography. For immunocomplex kinase assays, Aur-A was immunoprecipitated as described above and Sepharose bead-bound protein–antibody complexes were washed with kinase assay buffer. Beads were resuspended in kinase assay buffer and the assay was performed as described above, using recombinant His-Cofilin as the substrate.

2.6. Phosphopeptide analysis

Recombinant His-Aur-A or His-Aur-A^{K162M} was incubated with recombinant His-Cofilin in kinase assay buffer and cold ATP for 30 min at room temperature. Proteins were separated by SDS-PAGE and the polypeptide bands were visualized by staining with Coomassie Blue. Cofilin bands from both samples were excised from the gel and used for LC MS/MS with titanium oxide enrichment at the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale Cancer Center Mass Spectroscopy Resources) as previously described [8].

2.7. Dual label immunofluorescence

M12 cells (4×10^4) were seeded onto poly-L-lysine coated glass coverslips and transfected with Cofilin-RFP constructs 24 h later as described above. At 48 h post-transfection, cells were washed with PBS, fixed using 4% paraformaldehyde for 10 min at room temperature and permeabilized with 4% paraformaldehyde containing 0.2% Tween-20 for 10 min at room temperature. Permeabilized cells were blocked in blocking solution (10% goat serum, 0.2% Tween-20, 2% BSA in PBS) for 90 min at room temperature. Cells were stained with Phalloidin for F-actin, washed in sodium phosphate buffer and coverslips were mounted with DAPI Fluoromount G (Southern Biotech). For localization of Cofilin in mitotic cells with or without treatment with Aur-A inhibitor, M12 cells (3×10^4) were seeded onto poly-L-lysine coated coverslips and after 24 h treated with either 100 nM MLN8237 or DMSO for an additional 24 h. Next, cells were synchronized at G2/M phase and released into mitosis as described above. Coverslips were washed with PBS and fixed/permeabilized with 100% methanol for 10 min at −20 °C. Coverslips were blocked and treated with anti-Cofilin and anti-α-tubulin primary antibodies and anti-mouse AlexaFluor-488 and anti-rabbit Cy3 secondary antibodies. Cells were counterstained with DAPI and mounted as described above. For F-actin staining of MCF7 cells, 3×10^4 cells were seeded onto poly-L-lysine coated glass coverslips. Coverslips were washed with PBS and fixed with 4% paraformaldehyde and permeabilized with 4% paraformaldehyde containing 0.2%