Mechanisms of Signal Transduction:
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Critical Mediator for Aurora-A-induced 
Cellular Motility and Transformation by 
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Identification of V23RalA-Ser^{194} as a Critical Mediator for Aurora-A-induced Cellular Motility and Transformation by Small Pool Expression Screening*\(^{\text{S}}\)

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Human Aurora kinases have three gene family members: Aurora-A, Aurora-B, and Aurora-C. It is not yet established what the specificity of these kinases are and what signals relayed by their reactions. Therefore, we employed small pool expression screening to search for downstream substrates of Aurora-A. Interestingly, all of the identified Aurora-A substrates were resistant to serve as substrates for Aurora-B or Aurora-C, suggesting that these Aurora family members may have distinct substrate specificity for propagation of diverse signaling pathways, even though they share a conserved catalytic kinase domain. Of the candidate substrates, Aurora-A could increase the functional activity of RalA. Mutational analysis revealed that RalA-Ser^{194} was the phosphorylation site for Aurora-A. Ectopic expression of V23RalA-WT could enhance collagen I-induced cell migration and anchorage-independent growth in Madin-Darby canine kidney (MDCK) Aurora-A stable cell lines. In contrast, overexpression of V23Rala-S194A in MDCK Aurora-A stable cell lines abolished the intrinsic migration and transformation abilities of Aurora-A. To our knowledge, this is the first systematic search for the downstream substrates of Aurora-A kinase. Moreover, these results support the notion that Aurora-A may act in concert with V23RalA through protein phosphorylation on Ser^{194} to promote collagen I-induced cell motility and anchorage-independent growth in MDCK epithelial cells.

Aurora, an emerging family of serine/threonine kinases, has recently drawn intense attention because of its association with the development of human cancers and mitotic progression (for review see Refs. 1–4). Phylogenetic classification reveals three human members, Aurora-A, -B, and -C (1). These kinases are characterized by a conserved kinase domain at the C terminus, whereas the N-terminal domains are of variable lengths and share low sequence identity (5). It has been found that Aurora-A gene overexpression is associated with cancer cell lines, invasive carcinoma, and spindle abnormality. Ectopic expression of Aurora-A transforms Rat-1 and NIH3T3 cells (6, 7). The issue of the oncogenic potential of Aurora-A remains controversial, however, particularly in light of the recent report that overexpression of Aurora-A fails to induce oncogenic transformation in mouse embryonic fibroblasts (8). Overexpression of Aurora-B causes polyploidy and leads to genome instability, which is a major factor in the predisposition of tumor cells (9). However, less is known with respect to the role of Aurora-C. The key questions to be resolved relate to the identity of the downstream targets of these Aurora kinases, and whether Aurora family members may recognize distinct downstream targets, hence propagating diverse signaling pathways. To fully understand how a protein kinase regulates biological processes, it is imperative to identify its substrate(s). Very little information is available regarding the substrates of human Aurora family members, however. Currently, several potential substrates have been identified from different model organisms, such as: yeast (Ask1, Dam1, Spc34, Sli15, Ndc80, Ndc10, Cin8, and Histone H3) (10); Drosophila (dTACC) (11); Xenopus (Eg5 (12) and CPEB (13)); and human (TACC3 (14), TPX2 (15), MBD3 (16), CENP-A (17), p53 (18), and BRCA1 (19)). However, it is essential to identify the one or more phosphorylation sites of a given substrate to facilitate studies with phosphorylation site mutants to investigate the signals a kinase relay. Most of the above substrates are involved in mitotic progression, or spindle or centrosome regulation. For example, TPX2, a component of the spindle apparatus, is required for the targeting of Aurora-A to the spindle microtubules (15); whereas BRCA1, a multiple function protein, is involved in G2-M phase transition (19). In contrast, overexpression of Aurora-A could increase telomerase activity through c-myc in human ovarian and breast epithelial cells (20), supporting the notion that Aurora-A may participate in other cellular processes. Furthermore, a proposed consensus site (K/R)(X)(S/T)-(I/L/V), has been deduced for yeast Aurora kinase (Ipl1) phosphorylation (10). There is only one Aurora gene family member in yeast. By contrast, there are two in Drosophila and Xenopus, and three in humans. This
raises a series of unanswered questions, including whether the human counterparts of these identified substrates from different model organisms serve as the targets for all human Aurora family members or for just one of the Aurora kinases, and, whether the proposed consensus site for Ipl1 is indeed the bona fide substrate recognition motif for Aurora kinases in humans.

In this study, small pool expression screening, which has been used successfully to identify substrates for protein kinases (21, 22), was employed to identify potential Aurora-A substrates. The underlying concept is subdivision of the whole library into smaller pools to substantially increase clone abundance and the probability of detecting potential substrates for a given kinase in the pool. Moreover, this method makes it easier to rapidly isolate a single clone once a candidate substrate is identified, because, relative to large pools, small pools are less likely to contain multiple candidate cDNAs. Indeed, several potential substrates for Aurora-A were identified in this study. Among the identified substrates, two potential substrates, Ral-GDS and RalA, were of particular interest. Both are less likely to contain multiple candidate cDNAs. Indeed, small pools at least one protein band exhibiting a shift in electrophoretic mobility when incubated with WT but not KR form of Aurora-A, implicating these pools contained potential substrates. We then transformed these eleven pools respectively, isolated single clones, and tested with kinase reactions as described above. The cDNA clones of the [32P]methionine-labeled proteins (derived from single clones), which showed slowly migrated bands, were sequenced, and the different genes were identified. These substrates were then incubated with different protein kinases, including E. coli-expressed GST-Aurora-A/B, and baculovirus-expressed His-tagged Aurora-C (35). The substrates that exhibited electrophoretic mobility shift when incubated with various protein kinases were scored as positive as shown in Supplementary Table S1. To demonstrate whether protein phosphorylation but not other post-translational modifications caused the electrophoretic mobility shift, λ phosphatase (100 units per reaction, New England Biolabs) was used to verify this finding.

In Vitro Kinase Reaction—2 μg of purified GST-tagged RalA-(WT/S194A) or V23Ral-(WT/S194A) or RalB fusion proteins were incubated with purified recombinant GST Aurora-A proteins in the kinase reaction buffer (25 mM Tris HCl, pH 7.4, 10 mM MgCl2, 10 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol) coupled with 2.5 μCi of [γ-32P]ATP at 30 °C for 15 min. In general, the reaction volumes were 40–50 μl. Kinase reactions were terminated by adding SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The peptides were synthesized by using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry (36). After trypsin digestion, the peptide was cleaved from resin support with trifluoroacetic acid treatment. The purification of the synthetic peptide was conducted by reverse-phase high-performance liquid chromatography. The purity of each peptide was at least 90%. The molecular weight of each synthetic peptide was analyzed and confirmed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Equal amounts of each peptide (5 μg) were incubated with recombinant 0.2 μg of His-Aurora-A and 0.6 μg of His-Aurora-B (Upstate Biotechnology Inc.) proteins, respectively. The kinase reaction was performed in the kinase reaction buffer as described above with 2.5 μCi of [γ-32P]ATP at 30 °C for 5 min. In general, the reaction volumes were 20–30 μl. The reactions were then stopped by spotting on P81 ion exchange filter paper (Whatman), and the paper was washed with 1% phosphoric acid 20 min for three times as described earlier (37). CPM (counts per minute) values of each reaction were acquired via liquid scintillation analyzer (Packard). The phosphorylation extent of Aurora-A/B to various RalA peptides was normalized with RalA-S194-WT peptide as relative activity.

Cell Culture and Transient Transfection—293T and NIH3T3 cells were maintained at 37 °C in a 5% CO2/95% air environment incubator and grown in 10% DMEM medium supplemented with 10% fetal bovine serum (FBS) or calf serum and 100 μg/ml penicillin/streptomycin (Invitrogen) and 2 mM glutamine (Invitrogen). Additional sodium pyruvate (Invitrogen) was also supplemented for NIH3T3. Transient transfection of various constructs into 293T or NIH3T3 cells was performed with Lipofectamine (Invitrogen) according to the manufacturer's instructions. 48 h after transfection, the transfected cells were harvested for following experiments.
Preparation of Cell Lysates, Immunoprecipitation, and In Vitro Kinase Assay—To prepare cell-free lysates, cells were harvested, washed with phosphate-buffer saline, and lysed in extraction buffer, which was composed of 50% lysates buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin) and 50% immunoprecipitation washing buffer (10 mM HEPES, pH 7.6, 2 mM MgCl2, 50 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, and 40 mM β-glycerophosphate) as described earlier (38). Briefly, after incubation at 4 °C for 30 min, cellular debris was removed by centrifugation at 13,000 rpm for 30 min. Protein concentrations of total lysates (50–500 μg) were determined by counting all migrated cells of each clone 3B5, which were maintained at 37 °C in a 5% CO2/95% air environment. Cell lysates were incubated with antibodies against target epitopes and Protein A/G-agarose beads (Oncogene Research Product) to immunoprecipitate the target protein at 4 °C for 4 h. These immune complexes were washed three times with immunoprecipitation washing buffer as described above. The immunoprecipitated complex was then washed with indicated purified recombinant kinase in the kinase reaction buffer as described in previous section with 2.5 μCi [γ-32P]ATP at 30 °C for 15 min. In general, the reaction volumes were 40–50 μl. Kinase reactions were terminated by adding SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore Corp.) followed by autoradiography.

Ral Activation Assay—The Ral activation assay was performed as previously described (39). Briefly, NIH3T3 cells were transfected with RAL-A-WT (Upstate Biotechnology) and/or HA-V23RalA-(WT/S194A) with Lipofectamine (Invitrogen) following the manufacturer’s instruction. Cell lysates were prepared in buffer containing 10% glycerol, 2% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 20 mM NaF, 1 mM sodium vanadate, and 10 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin (Sigma). 1 ng of proteins from each sample was precleaned with glutathione beads (Amersham Biosciences) and then incubated with 20 μl of glutathione S-transferase (GST)-Ral binding domain beads according to the manufacturer’s instruction (Upstate Biotechnology) for 1 h at 4 °C. The beads were then washed three times with 1× RAB buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, and 0.5 mM dithiothreitol). Samples were subjected to 12% SDS-PAGE electrophoresis, transferred to PVDF membrane, and Western blotted with anti-RalA (Upstate Biotechnology Inc.) or anti-HA monoclonal antibody (3F10, Roche Applied Science).

Establishment of Stable Clones in MDCK 3B5 Cells—MDCK cells, clone 3B5, were maintained at 37 °C in a 5% CO2/95% air environment incubator and grown in DMEM supplemented with 10% heat-inactivated FBS and 100 μg/ml penicillin/streptomycin (Invitrogen). Subconfluent MDCK cells were transfected with various combinations of HA-tagged V23RalA(WT/S194A) and FLAG-tagged Aurora-A(WT/KR) constructs and dithiothreitol (Invitrogen) according to the manufacturer’s instructions. MDCK cells stably expressing various constructs were selected in medium containing 800 μg/ml G418 (Calbiochem). An aliquot of 104 cells of each stable clone or parental MDCK cells was suspended in 400 μl of DMEM containing 0.5% FBS and seeded onto a layer of 10% FBS-DMEM containing 0.5% Seakem-agarose and in a 60-mm dish. 2 ml of DMEM containing 10% FBS was then added. The plates were incubated at 37 °C in a 5% CO2/95% air environment incubator with replaced medium every 3 days. After 4 weeks, the colony numbers of each clone from three independent experiments were stained with crystal violet (Sigma), counted, and normalized with parental MDCK cells as relative ratio.

RESULTS

Identification of Downstream Substrates of Aurora-A by Small Pool Expression Screening—Small pool expression screening was used to identify the downstream substrates of Aurora-A protein kinase. Briefly, we transfected and translated small pools of human placenta cDNA library in vitro in the presence of [35S]methionine as described under “Experimental Procedures.” The [35S]methionine-labeled protein pools were incubated with either purified GST-wild-type (WT) or catalytically inactive (KR) recombinant Aurora-A and analyzed by SDS-PAGE followed by autoradiography. In the screening for electrophoretic mobility shift on SDS-PAGE, 11 out of 342 cDNA small pools tested exhibited mobility shift changes in Aurora-A-WT, but not Aurora-A-KR-treated protein pools. One of the positive pools is shown in Fig. 1A. Subsequently, treatment with a phosphatase abolished the slowly migrating mobility shift on SDS-PAGE, indicating that the electrophoretic mobility shift was caused by protein phosphorylation (data not shown). All positive pools were retransformed into E. coli, and the individual cDNA clones were isolated and tested with kinase reactions as described under “Experimental Procedures.” We subsequently sequenced those cDNA clones that tested positive and identified nine non-redundant cDNA clones. Fig. 1B summarizes the findings and showed that nine [35S]methionine-labeled single clones exhibited electrophoretic mobility shifts on SDS-PAGE when incubated with Aurora-A-WT. Several of the identified substrates, such as c-Fos and FLJ10877, were among those that are substrates of Aurora-A. This result is shown in Supplemental Table I. It should be noted that (a) phosphorylation does not always lead to an electrophoretic mobility shift (the assay, therefore, can detect only a subset of potential substrates), and (b) our screens were not considered saturated, because not all of the positive clones were isolated multiple times, and it is therefore not surprising that the identified substrates did not include any previously identified molecules as described in the introduction. These results suggest that more potential substrates for Aurora-A remain to be uncovered. Interestingly, the possible functions of these potential substrates cover a wide range of biological responses, including Ras signaling, transcription, and translation control (Supplemental Table I), suggesting the role of Aurora-A might not be as limited as previously thought.

V23RalA-Ser194 Mediates Aurora-A Oncogenic Activity
Biochemical Characterization of the Substrate Specificity among Aurora Family Kinases—Several identified substrates for Aurora-A, such as c-fos and ribosomal protein S6, are widely known to serve as the substrates for a number of protein kinases (41, 42). To fully examine whether these identified substrates have any specificity toward Aurora-A family members, we then incorporated the [35S]methionine-labeled substrates with each Aurora family kinase. First, we investigated whether Aurora-B or Aurora-C could phosphorylate the substrates identified from this study for Aurora-A. Surprisingly, both Aurora-B and Aurora-C kinases, which both were active (Fig. 2A), could not cause the mobility shift of any of the substrates tested, at least in our assay condition. These results, however, did not rule out the possibility that Aurora-B and Aurora-C may need accessory proteins to enhance their kinase activities to efficiently phosphorylate the protein substrates. Two examples were shown in Fig. 2 (B and C). [35S]Methionine-labeled FLJ10877 fis or Ral-GDS could serve as substrates for Aurora-A WT, but not for Aurora-A KR, Aurora-B, or Aurora-C, based on the mobility shift on SDS-PAGE as described earlier. Alternatively, to enhance the assay sensitivity, HA-tagged Ral-GDS was expressed in 293T cells. Cell lysates were immunoprecipitated with HA-antibody followed by kinase assay in the presence of [γ-32P]ATP (Fig. 2C, middle panel) or Western blot (Fig. 2C, bottom panel). The result again demonstrated that Ral-GDS could serve as a substrate for Aurora-A WT but not other Aurora forms tested. In fact, all three Aurora kinases seemed to have different specificity toward different exogenous substrates (Fig. 2A). For example, Aurora-B caused the slightly different mobility shift of histone H1 on SDS-PAGE in the presence of [γ-32P]ATP and Aurora-C could phosphorylate p16 well (35) but not histone H1 and myelin basic protein (Fig. 2A). Taken together, none of the identified substrates served as substrates for Aurora-B and Aurora-C, suggesting that the Aurora family kinases might have different substrate specificity to propagate the diverse signaling pathway despite sharing considerable sequence homology in their kinase domains at the C terminus.

Ectopic Expression of Aurora-A Activates RalA—Among the identified substrates, RalA is of interest for its involvement in cell motility, anchorage-independent growth, and many other events as described earlier. Thus, we decided to test what would be the functional relationship between RalA and Aurora-A. We tested whether Aurora-A could modulate endogenous RalA activity by using a GST-RalBP1-RBD immunoprecipitation assay (39), which will pull down the active form of RalA, as described under “Experimental Procedures.” Ectopic expression of FLAG-Aurora-A WT in NIH3T3 cells led to the increase of endogenous RalA activity in a dose-dependent manner,
was illustrated at the described under "Experimental Procedures." The endogenous Ral activity was determined by using GST-RalBP1-RBD immunoprecipitation assay as described under "Experimental Procedures." The endogenous Ral activity was illustrated at the bottom panel (as shown by asterisks).

whereas overexpression of FLAG-Aurora-A-KR down-regulated RalA activity (Fig. 3). These results suggest that the Aurora-A kinase activity is attributable to the functional activity of RalA.

Aurora-A Phosphorylates RalA on Ser

FIG. 3. Ectopic expression of Aurora-A activates RalA. Different amounts (1, 2, and 5 μg) of FLAG-Aurora-A-WT or FLAG-Aurora-A-KR were transfected into NIH3T3 cells. After 48 h, the cells were collected and lysed. The top panel showed the protein expression level of FLAG-Aurora-A-WT/KR as examined by Western blotting using anti-FLAG antibody. 1 mg of cell extracts was subjected to Ral activity determination by using GST-RalBP1-RBD immunoprecipitation assay as described under "Experimental Procedures." The endogenous Ral activity was illustrated at the bottom panel (as shown by asterisks).

V23RalA-Ser194 Mediates Aurora-A Oncogenic Activity

Identification of the Specificity Determinants for Aurora-A—The amino acids around the phosphorylation site play a pivotal role in recognition by distinct protein kinases. However, it is not known whether human Aurora family kinases could recognize unique phosphorylation site consensus sequences (herein referred to as substrate specificity determinants), despite a proposed consensus site, (K/R)(X)(S/T)-(I/L/V), being deduced for yeast Ipl1 phosphorylation (10). Therefore, the RalA-S194 peptide (189KKKKSLAKRI199) was synthesized and tested for serving as a substrate for recombinant Aurora-A/B. An in vitro kinase assay indicated that Aurora-A, but not Aurora-B, efficiently phosphorylated RalA-Ser194 peptide with an apparent Km of 0.47 mM, despite the fact that both kinases were active and exhibited similar activity by using myelin basic protein as a substrate (Fig. 5A), further supporting the idea of distinct substrate selection between Aurora-A and Aurora-B.

To further elucidate the specific residues involved in substrate recognition of Aurora-A, we applied alanine-scanning mutagenesis to identify specific side chains that may strongly modulate substrate specificity toward Aurora-A. Alanine was chosen as the replacement residue, because it eliminates the side chain beyond the β carbon. This approach will therefore generate a systematic set of mutant peptides that can be readily assayed by quantitative phosphorylation analysis. This approach was used to generate a series of mutant peptides of RalA-Ser194. 189KKKKSLAKRI199 by replacing each residue with alanine. A total of ten single alanine mutant peptides were synthesized, and their relative extent of phosphorylation was determined by incubating with recombinant His-Aurora-A in the kinase reaction buffer containing [γ32P]ATP. Substituting Ala for Ser at the center (Ser194) of the 189KKKKSLAKRI199 peptide abolished the recombinant His-Aurora-A-mediated phosphorylation, suggesting the Ser residue is indeed the phosphorylation site for Aurora-A in this peptide. More importantly, substituting Arg192 and Leu195 with Ala individually completely eliminated Aurora-A phosphorylation (Fig. 5B). On the contrary, replacing other positively charged residues with Ala individually in the wild-type peptide did not reduce the phosphorylation status by Aurora-A, suggesting RXSL is the substrate specificity determinant for Aurora-A in 189KKKKSLAKRI199 peptide.

V23RalA-S194A Serves as a Loss-of-Function Mutant to Block Aurora-A-mediated Epithelial Cell Migration—Elevated gene expression of Aurora-A had been reported to correlate with invasion and rates of metastasis of human bladder cancer (43). This observation raises the possibility that Aurora-A might be involved in cell migration. If this is indeed the case, what would be the downstream signal(s) mediated by Aurora-A? Among the identified substrates, RalA exhibits several characteristics similar to Aurora-A as described in the introduction. To delineate the biological effects of Aurora-A and RalA in cellular migration and transformation in epithelial cells, we used MDCK renal epithelial cells as our model system, which is a suitable model for assaying cell transformation and migration process (44–46), to establish various combinations of Aurora-ARalA (WT/S194A) stable clones in MDCK cells. However, these stable clones did not exhibit any significant difference in cell growth, migration, and anchorage-independent growth ability (data not shown). Subsequently, we used the constitutively active form of RalA, namely V23RalA, in our assay to address the possible connection and biological role of Aurora-A and RalA. We first determined whether V23RalA could be phosphorylated by Aurora-A. In vitro kinase assay indicated that GST-V23RalA, but not GST-V23RalA-S194A, could be phosphorylated by recombinant Aurora-A (Fig. 6A). Subsequently, we established various combinations of Aurora-A/V23RalA (WT/S194A) stable clones in MDCK cells. The expression levels of each combination of exogenous FLAG-tagged Aurora-A-WT/KR
and HA-tagged V23RalA-WT/S194A were verified via Western blotting (Fig. 6B), and these stable clones were used in the migration and soft agar assays in the following studies.

To determine whether Aurora-A might exhibit potential to promote cell motility, 5 × 10³ parental or Aurora-A-expressing MDCK cells were seeded as a monolayer on top of a Transwell insert. No obvious cell motility was observed in Aurora-A-WT-expressing MDCK cells, whereas both MDCK parental cells and Au-
rora-A-KR-expressing MDCK cells had basal motility capacity (Fig. 7A). Fig. 7B shows the quantitative measurement of at least five different experiments as described under “Experimental Procedures.” The data suggest that Aurora-A kinase activity is essential for epithelial cell migration, implicating that downstream targets of Aurora-A might play a role in transmitting Aurora-A-mediated cell migration.

We next determined whether RalA might function in Aurora-A-induced cell motility. Cell migration assays were employed with MDCK cell lines expressing Aurora-A-WT/V23RalA-WT, Aurora-A-WT/V23RalA-S194A, V23RalA, or V23RalA-S194A. Both V23RalA- and V23RalA-S194A-expressing MDCK cells had basal migration ability, similar to that of control MDCK parental cells (Fig. 7B). However, coexpression of Aurora-A-WT and V23RalA in five different MDCK cells resulted in a significant increase in mobility compared with that of control MDCK parental cells (Fig. 7B). However, coexpression of Aurora-A-WT and V23RalA in five different MDCK cells resulted in a significant increase in mobility compared with that of Aurora-A-expressing MDCK cells. Moreover, the migration capacity of MDCK cells coexpressing Aurora-A-WT and V23RalA-S194A was almost reduced to a baseline level (Fig. 7, A and B). Together, the findings suggest that the Aurora-A-V23RalA signaling module might regulate the epithelial cell migration in the presence of collagen I through protein phosphorylation of V23RalA on Ser194 and V23Ral-S194A may serve as a loss-of-function mutant to block Aurora-A-mediated epithelial cell migration.

V23RalA-S194A Serves as a Loss-of-function Mutant to Block Aurora-A-mediated Anchorage-independent Growth—Overexpression of Aurora-A in murine NIH3T3 or Rat1 cells was manifest as oncogenic transformation and tumorigenesis (6, 7) but failed to possess oncogenic potential in mouse embryonic fibroblasts (8). Therefore, we decided to examine the role of Aurora-A in oncogenic transformation in epithelial cells by using an anchorage-independent growth assay (30), a critical phenomena for cell oncogenic transformation. 10⁴ parental, Aurora-A-WT, or Aurora-A-KR MDCK stable cells were plated in soft agar and scored for growth efficiency after 4 weeks. As shown in Fig. 8A, cells expressed Aurora-A-WT could form colonies in soft agar, in contrast to the lack of growth of parental or Aurora-A-KR cells, supporting the role of Aurora-A in oncogenic potential.

We then evaluated whether V23RalA, or specifically V23RalA-Ser194, participates in Aurora-A-mediated cell anchorage-independent growth ability, because RalA has been reported to be required for anchorage-independent growth by overexpression dominant negative form of RalA (24) or siRNA (30) and serves as a downstream substrate to modulate the role of Aurora-A in epithelial cell migration. In agreement with a previous report (24), which showed that cells stably expressed 72LRalA could not form a colony in a soft agar assay, Fig. 8B shows that V23RalA alone could not induce cell transformation. In contrast, coexpression of V23RalA with Aurora-A-WT significantly increased the number of colony formation in soft agar assay (Fig. 8, A and B), implying that V23RalA-Ser194, again, plays a critical role in Aurora-A-mediated cellular transformation process. In summary, ectopic expression of Aurora-A may act in concert with V23RalA through protein phosphorylation on V23Ral-Ser194 to promote collagen I-induced cell motility and anchorage-independent growth in MDCK epithelial cells.

DISCUSSION

Identification of downstream substrates of a protein kinase is an essential step to provide better understanding of its uncharted functions. Overexpression of Aurora-A gene was
FIG. 7. Effects of Aurora-A/V23RalA MDCK stable clones in collagen I induced cell migration. A, $5 \times 10^3$ cells of MDCK (vector control) or various MDCK stable clones were seeded into the top of a Transwell insert, where collagen I was coated to the bottom of the dish. 22 h later, the cells on the topside were scraped, and the cells that migrated to the bottom were fixed and stained with crystal violet. The photographic results of MDCK, Aurora-A-WT#10, Aurora-A-WT/V23RalA#2, and Aurora-A-WT/V23RalA-S194A#22 stable clone were showed (200×). B, the relative -fold migration of each stable clone was normalized with MDCK cells and represented as diagrammatic results.

FIG. 8. Effects of Aurora-A/V23RalA MDCK stable clones on anchorage independent cell growth ability. A, $1 \times 10^4$ cells of MDCK (vector control) or various stable clones were plated in soft agar incubated in 37 °C, 5% CO₂/95% air environment incubator for 28 days. Subsequently, the colonies formed in the agarose were stained with crystal violet and counted. The photographic results of MDCK, Aurora-A-WT#10, Aurora-A-WT/V23RalA#9, and Aurora-AWT/V23RalA-S194A#22 stable clones were represented. B, all colonies of each clone were counted and normalized with MDCK cells as relative -fold and represented as diagrammatic results.
found in many tumors (47) and had been shown to be oncocogenic in murine NIH3T3 or Rat1 cells (6, 7), although no oncocogenic potential in mouse embryonic fibroblast could be demonstrated (8). This study aimed to elucidate the role of Aurora-A in a malignant transformation process through the identification of its downstream substrates. By performing large scale biochemical analysis, we successfully identified nine candidate substrates for Aurora-A and revealed previously unidentified features correlating with the functionality of Aurora-A. This is the first report of a systematic search for the downstream substrates of Aurora-A kinase. In this study, we demonstrated: (a) Aurora-A has distinct substrate specificity compared with Aurora-B/C; (b) the newly identified potential substrates cover a wide range of biological responses, suggesting the role of Aurora-A might not be as limited as previously thought; (c) Aurora-A phosphorylates RalA on Ser194 and the substrate specificity determinant of RalA-S194 peptide is RXSL; and (d) Aurora-A stable clones in MDCK promote collagen I-induced cell motility and anchorage-independent growth ability through phosphorylation on V23RalA-Ser194. These findings highlight the utility of comprehensive biochemical analysis to elucidate the functionality of Aurora-A.

By using alanine-scanning mutagenesis on the RalA-S194 peptide, in vitro Aurora-A kinase assays demonstrated that basic residues at P+2 and a hydrophobic residue at P−1 were important for efficient phosphorylation of the serine at P0, suggesting that RXSL is the substrate specificity determinant for Aurora-A. The finding is similar to the substrate specificity determinant for yeast Aurora kinase-Ipl1, (K/R)(X)S/T/I/L/V) (10), suggesting that it is likely an evolutionary conservation between Aurora-A and Ipl1 in substrate recognition. In fact, several potential substrates for Aurora-A, such as TACC3 (14), Xenopus Eg5 (12), RalGDS, osteopontin, and FLJ10877 fis, for which the phosphorylation motif has not yet been determined, contain the RXSL motif and might serve as a recognition target for Aurora-A. On the other hand, several reported phosphorylation sites, such as CPEB (48), p53 (18), and BRCA1 (19), did not have such a motif, making the implication that additional substrate specificity determinants for Aurora-A remain to be identified, which could be deduced by systematic analysis with the expansion of a larger dataset followed by kinetic analysis.

Ral GTPase contains two homologous genes, RalA and RalB, in human. RalA had been shown to play an essential role in epidermal growth factor-mediated cell motility (29) and be required for the anchorage-independent growth ability by overexpression of dominant negative RalA(28N) mutant (24). Knockdown RalA or RalB gene by small interference RNA showed that RalA gene is dispensable for survival but is required for anchorage-independent proliferation in human cancer cells (for example, MCF7 and SW480), whereas RalB gene is required for suppression of apoptosis in tumor cell lines (30). In addition, our result showed that Aurora-A phosphorylated RalA, but not RalB, and activated RalA. These data together suggest that RalA and RalB may have distinct signaling pathways, despite the fact that genes RalA and RalB share 80% sequence identity. Furthermore, V23RalA (constitutively active form) and Aurora-A stable clones could promote collagen I-induced migration and anchorage-independent growth ability, suggesting that Aurora-A might cooperate with RalA upstream regulators like Ral-GDS to induce RalA oncogenic ability.

Moreover, we examined whether Aurora-A might also play a role in cell invasion via V23RalA. We analyzed all of the stable clones established in this study by using a Matrigel invasion assay as described previously (49). However, there was no difference between parental and various MDCK stable clones tested (data not shown). In contrast, pretreatment with hepa-
tocyte growth factor (49), Aurora-A/V23RalA stable clones exhibited higher invasion ability than other clones tested (data not shown). Together, overexpression of V23RalA-S194A in MDCK Aurora-A stable cell lines abolished Aurora-A-mediated collagen I-induced migration, hepatocyte growth factor-induced invasion, and anchorage-independent growth ability of these cells. These data suggest that the intrinsic migration, invasion, and transformation abilities of Aurora-A might be mediated by phosphorylation of RalA-Ser194 and support an essential role of RalA in the cellular transformation process (30). Our finding also raises the possibility that the role of Aurora-A, other than mitotic regulation, might also participate in a Ras signaling pathway through RalA phosphorylation.

It had been proposed that ectopic expression of Aurora-A might cause formation of multiple centrosomes and subsequent cellular transformation in HeLa or NIH3T3 cells (7, 50, 51). In contrast to these reports, the ratio of multiple centrosomes in various Aurora-A and/or V23RalA MDCK stable clones was relatively low (<10% per clone), and no significant difference was observed. This discrepancy might be due to different cell types, or Aurora-A might mediate cellular transformation via RalA in a multiple-centrosome-independent manner. To support this speculation, ectopic expression of the N-terminus-truncated Aurora-A-(121–403) was able to transform cells but lacked the ability to induce multiple centrosomes, suggesting that Aurora-A-mediated centrosome amplification and cellular transformation might proceed through distinct mechanisms (51). Another important molecule in Aurora-A-mediated cellular transformation is p53 (50, 51). Previous reports showed that Aurora-A could transform cells in a higher degree when p53 was defective. In addition, Aurora-A could phosphorylate p53, leading to its ubiquitination by Mdm2 and proteolysis (18), suggesting an antagonistic relationship between Aurora-A and p53 in the cellular transformation process. Whether p53 plays a role in the Aurora-A/RalA signaling network remains to be determined.

In summary, Aurora-A-mediated cellular transformation depends on its intrinsic kinase activity, implicating that the downstream substrates of Aurora-A play prerequisite roles in this process. Identification of RalA as one of the downstream substrates of Aurora-A provides a novel signaling network of Aurora-A in cell migration and oncogenic transformation through V23RalA-Ser194 phosphorylation.

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