Mechanisms of Signal Transduction:

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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*[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 G_2-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 process. 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

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table SI.

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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 involved in the Ras-primed cell transformation pathway (23). The activation of RalA enhances the transforming activities of RasH and Raf (24). It has been demonstrated that RalA (or its constitutively active forms G23V and Q72L) are involved in multiple signaling pathways, including: the activation of phospholipase D1 and Src kinase (25, 26); vesicle transport (27); filopodia formation (28); epidermal growth factor-induced cell motility (29); and anchorage-independent proliferation of MCF-7 and SW480 cells (30). Moreover, Ral-GDS[1] and Ca^{2+}/calmodulin are known to be by far the best-known regulators of RalA (31, 32). Whether other proteins are involved in the regulation of RalA remains unclear, however.

In this study, nine potential substrates, spanning a broad range of biological responses, were identified based upon electrophoretic mobility shift on SDS-PAGE. Surprisingly, none of the identified substrates serve as substrates for Aurora-B and Aurora-C, suggesting that Aurora family members might have distinct substrate specificity, despite the fact that they share a conserved catalytic kinase domain. Furthermore, Aurora-A phosphorylates RalA on Ser^{194}, and Ser^{194} phosphorylation is critical for the activation of RalA. Ectopic expression of Aurora-A promotes collagen I-induced cell motility and anchorage-independent growth ability through phosphorylation on V23RalA-Ser^{194}. These findings suggest that elaboration of Aurora-A phosphorylation of RalA may provide mechanistic insights into the oncogenesis of Aurora-A.

EXPERIMENTAL PROCEDURES

DNA Constructs—Plasmids encoding the full-length *Aurora-A* and *RalA* were constructed in pCMV2-FLAG, HA-pcDNA3.1, or GST-vector, respectively. The kinase-dead mutant of *Aurora-A* and S138A, S183A, S194A (three phosphorylation site mutants), or G23V mutants for *RalA* were generated by site-directed mutagenesis (Stratagene) employing *Aurora-A* or *RalA* as the template.

Expression and Purification of GST Fusion Proteins—GST-tagged Aurora-A-(WT/KR), Aurora-B-(WT/KR), RalA, and RalB fusion proteins were expressed in *Escherichia coli* strain BL21(DE3). The fusion proteins were then purified by glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer's instruction and stored at −80 °C.

Small Pool Expression Screening—Small pool expression screening (22, 33) was used to identify the downstream targets of Aurora-A protein kinase, and we adopted the detailed protocol published previously (22, 33). Briefly, we have subdivided human placenta cDNA library (OriGene) into 342 small pools, which contain 200–300 clones instead of 100,000 clones per pool. Plasmids were isolated from each pool and then served as templates for *in vitro* transcription and translation (TnT Quick Coupled Transcription/Translation Systems from Promega) in the presence of [^{35}S]methionine. The procedures were according to the manufacturer's instruction, except that we added 0.5 μg of plasmid per reaction, and the reaction volume was scaled down to 5 μl. The *in vitro* kinase assay was described previously (34). The [^{35}S]methionine-labeled protein pools (5 μl) were incubated with either wild-type (WT) or catalytically inactive (KR) recombinant GST-Aurora-A in the kinase reaction buffer (25 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 100 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol) at 30 °C for 10 min. In general, the reaction volumes were 40–50 μl. If there is a potential substrate presented in a protein pool, an electrophoretic mobility shift caused by protein phosphorylation would be observed when protein samples are incubated with WT but not with KR form of Aurora-A. To increase the possibility of separating the phosphorylation and non-phosphorylation forms, reaction mixtures were loaded side by side on a 20-cm SDS-PAGE (12% polyacrylamide gel) and changing the acrylamide:bis ratio from 29:1 to 100:1. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.) followed by autoradiography. Among the 342 cDNA small pools screened, 11 pools contained 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 [^{35}S]methionine-labeled proteins (derived from single clones), which showed slowly migrated bands, were sequenced, and nine 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 SI. 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 $MgCl_2$, 10 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol) coupled with 2.5 μCi of [γ-^{32}P]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 (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (36). After completion of the peptide synthesis, 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 of μCi [γ-^{32}P]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% CO_2/95% air environment incubator and grown in DMEM medium supplemented with 10% heat-inactivated 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.

[1] The abbreviations used are: GDS, guanine nucleotide dissociation stimulator; GST, glutathione *S*-transferase; PVDF, polyvinylidene difluoride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MDCK, Madin-Darby canine kidney cells; WT, wild type.

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 Na_3VO_4, 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 $MgCl_2$, 50 mM NaCl, 5 mM EGTA, 0.1% Triton X-100, and 40 mM β-glycerolphosphate) 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 were determined using BCA Protein Assay reagents (Pierce). 500 μg of total cell lysates was 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 incubated with indicated purified recombinant kinase in the kinase reaction buffer as described in previous section with 2.5 μCi of [γ-^{32}P]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 previous described (39). Briefly, NIH3T3 cells were transfected with FLAG-Aurora-A-(WT/KR) 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-HCl, pH 7.4, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM NaF, 1 mM sodium vanadate, and 10 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin (Sigma). 1 mg 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 $MgCl_2$, 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% CO_2/95% 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 with Lipofectamine (Invitrogen), according to the manufacturer's instructions. MDCK cells stably expressing various constructs were selected in medium containing 800 μg/ml G418 (Calbiochem). An individual clone was picked up and analyzed for exogenous Aurora-A or V23RalA expression by Western blotting. Each selected stable clone was lysed with RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris, pH 8.0, and 10 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin). After incubation at 4 °C for 30 min, cellular debris was removed by centrifugation. Equal amounts of total lysates (50 μg) were subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and probed with 1:2000 dilution of anti-FLAG (M5, Sigma) or anti-HA (3F10, Roche Applied Science) antibody followed by incubation with secondary antibodies conjugated to horseradish peroxidase, and developed using the ECL system (Amersham Biosciences).

Migration and Invasion Assay—Migration and invasion assays of various MDCK stable clones were evaluated via 24-well Transwell (8-μm pore size polycarbonate membrane, Costar) chambers. For migration assay, the chambers were prepared by precoating with 30 μg of collagen I to the under surface of the membrane at room temperature for 30 min. The collagen I solution was then discarded, and the chambers were air dried for 20 min. For migration assay, 5×10^3 cells of various MDCK stable clones were suspended in 400 μl of DMEM containing 10% FBS and were seeded to the upper chamber, whereas 600 μl of DMEM containing 10% FBS was added to the outer side of the chamber. After being cultured in a 37 °C, 5% CO_2/95% air environment for 20 h, cells on the upper surface of the membrane were removed by a cotton tip applicator, and migratory cells on the lower membrane surface were fixed by methanol and stained with Giemsa (Sigma). Cell migration values were determined by counting all migrated cells of each stable clone under a phase-contrast microscope (200× magnitude) on five independent membranes and then normalized with parental MDCK cells as relative ratio. For invasion assay, 117 μg of Matrigel (BD Bioscience) was added to the upper surface of the membrane and allowed for gelling at 37 °C for overnight. 1 h before invasion assay, the collagen I was coated as described above. 1×10^4 cells in 400 μl of DMEM containing 0.5% FBS were seeded to the upper chamber, and 600 μl of DMEM containing 10% FBS was added to the outer side of the chamber. The following procedures for invasion assay were the same as in the migration assay described above.

Anchorage-independent Growth Assay—Anchorage-independent growth ability of various MDCK stable clones was determined by assessing colony formation efficiency in the soft agar system as in previous reports (40). Briefly, 1×10^4 cells of each stable clone or parental MDCK cells were suspended in 3 ml of 10% FBS-DMEM containing 0.3% Seakem-agarose (Cambrex BioScience Rockland). The suspension was then added 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% CO_2/95% 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 then 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 transcribed and translated small pools of human placenta cDNA library *in vitro* in the presence of [^{35}S]methionine as described under "Experimental Procedures." The [^{35}S]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. 1*A*. Subsequently, treatment with λ 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. 1*B* summarizes the findings and showed that nine [^{35}S]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 fis, exhibited significant electrophoretic mobility shifts, raising the possibility that there are multiple phosphorylation sites presented in these substrates. The comprehensive list of the identified substrates and their biological characteristics (annotation, as assigned by Gene Ontology (www.geneontology.org/), 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.

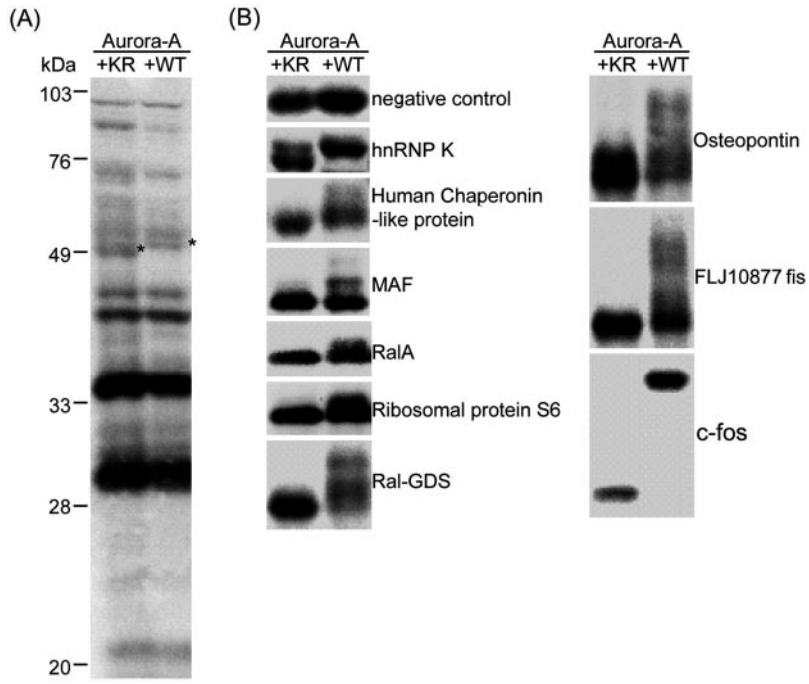


FIG. 1. **Identification of the downstream substrates of Aurora-A by small pool expression screening.** *A*, the [^{35}S]methionine-labeled protein pools were incubated with wild-type (*WT*) and catalytically inactive (*KR*) recombinant GST-Aurora-A, respectively. In this particular pool, there was a potential substrate present as illustrated by the electrophoretic mobility shift (as shown by *asterisks*) in the Aurora-A-WT- but not Aurora-KR-treated lane on SDS-PAGE. *B*, the positive pools were retransformed. Each single clone was analyzed by kinase reaction followed by autoradiography until the potential substrate was identified. Nine [^{35}S]methionine-labeled proteins exhibited the electrophoretic mobility shift in the Aurora-A-WT- but not Aurora-A-KR-treated lane.

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 incubated the [^{35}S]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. 2*A*), 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*). [^{35}S]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-^{32}P]ATP (Fig. 2*C*, *middle panel*) or Western blot (Fig. 2*C*, *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. 2*A*). For example, Aurora-B caused the slightly different mobility shift of histone H1 on SDS-PAGE in the presence of [γ-^{32}P]ATP and Aurora-C could phosphorylate p16 well (35) but not histone H1 and myelin basic protein (Fig. 2*A*). Taken together, none of the identified substrates served as substrates for Aurora-B and Aurora-C, suggesting that the Aurora family kinases might have distinct 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

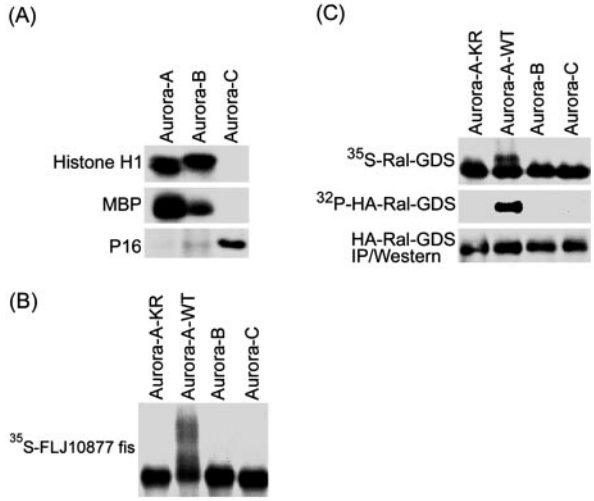


FIG. 2. **Aurora-A exhibits distinct substrate specificity compared with Aurora-B/C.** *A*, recombinant Aurora-A, Aurora-B, and Aurora-C were incubated with histone H1, myelin basic protein (*MBP*), and p16, respectively, as an exogenous substrate in the presence of [γ-^{32}P]ATP to ensure that the input of Aurora-A kinase activity was equivalent to that of Aurora-B, and Aurora-C was active. *B* and *C*, [^{35}S]methionine-labeled FLJ10877 fis or Ral-GDS was incubated with Aurora-A (WT and KR forms), Aurora-B, and Aurora-C, respectively. The electrophoretic mobility up-shift in the Aurora-A-WT-treated lane on SDS-PAGE indicates protein phosphorylation. *C*, HA-tagged Ral-GDS was transiently transfected into 293T cells for 48 h. Cell lysates were immunoprecipitated with HA-monoclonal antibody. The immunoprecipitants were incubated with Aurora-A (WT and KR forms), Aurora-B, and Aurora-C, respectively, in the presence of [γ-^{32}P]ATP. The reaction mixture was subjected to SDS-PAGE, transferred to PVDF membrane followed by autoradiography (*middle panel*), or Western blot analysis with anti-HA antibody (*bottom panel*).

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,

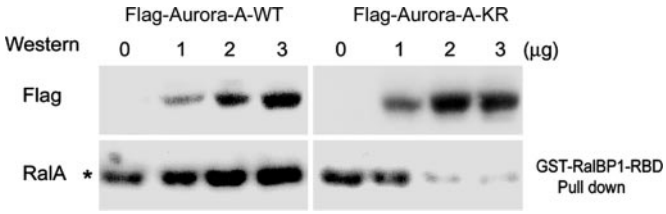


FIG. 3. **Ectopic expression of Aurora-A activates RalA.** Different amounts (1, 2, and 3 μg) of FLAG-Aurora-A-WT or FLAG-Aurora-A-KR were transfected into NIH3T3 cells. After 48 h, the cells were collected and lysed. The *top panel* showed the protein expression level of FLAG-Aurora-A-WT/KR as examined 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*).

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^{194}—Fig. 1*A* shows that [^{35}S]methionine-labeled RalA exhibited electrophoretic mobility up-shift on SDS-PAGE when incubated with Aurora-A-WT, but not Aurora-A-KR. However, the data were not tested for phosphorylation by Aurora-A in a purified system, raising the possibility that some of the potential substrates identified in the small pool expression screening were phosphorylated indirectly. Therefore, we switched the following kinase assays in a purified recombinant protein or peptide system.

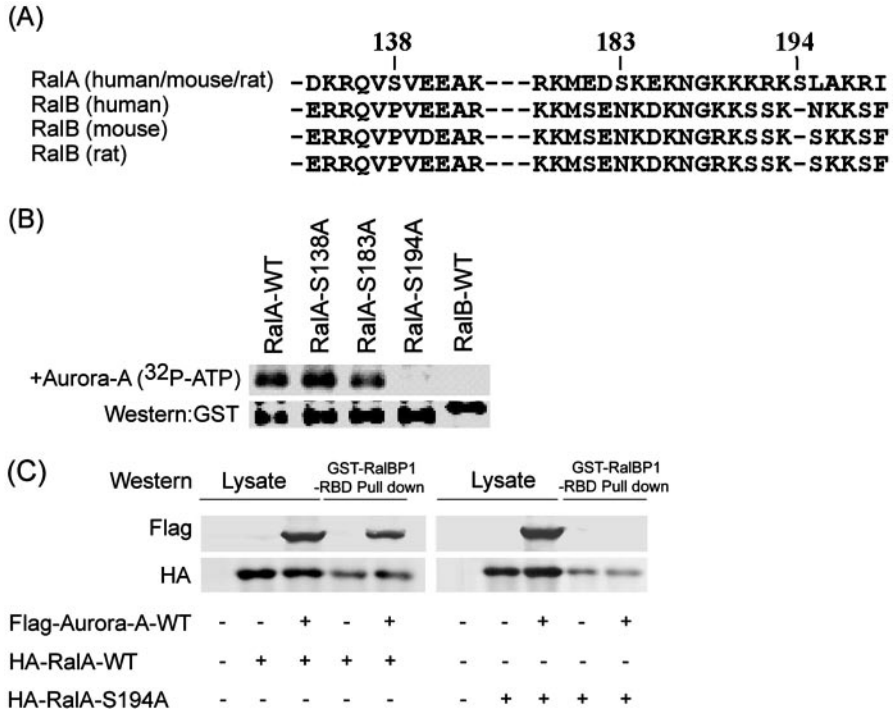
The Ral family contains two members: *RalA* and *RalB*, which share ~80% sequence identity. Because RalA served as a substrate for Aurora-A, we took the comparative genomics approach and asked whether RalB could serve as an Aurora-A substrate. To enhance the assay sensitivity, RalA and RalB were expressed in *E. coli* as GST fusion proteins, partially purified, and incubated with Aurora-A in the presence of [γ-^{32}P]ATP. The result showed that recombinant RalB failed to serve as a substrate for Aurora-A *in vitro* (Fig. 4*B*). This finding prompted comparison of the *RalA* and *RalB* serine/threonine sequences, with variation in only three serine amino acids when comparing the two (Fig. 4*A*). To determine the possible contribution of these three serine residues in RalA, we replaced individual serine residues with alanine and expressed them as GST fusion proteins in *E. coli*. An *in vitro* kinase assay indicated that RalA-Ser^{194} was the only site phosphorylated by recombinant Aurora-A (Fig. 4*B*).

To further characterize the possible interaction between Aurora-A and RalA, 293T or NIH3T3 cells were cotransfected with FLAG-tagged Aurora-A-(WT or KR) and HA-tagged RalA-(WT or S194A). Cells were lysed and immunoprecipitated with their respective epitope antibodies, and the presence of binding proteins was determined by Western analysis. However, results indicated that Aurora-A and RalA formed a very weak complex (data not shown). On the other hand, in NIH3T3 cells cotransfected with Aurora-A and RalA, the two proteins coimmunoprecipitated in experiments using the GST-RalBP1-RBD immunoprecipitation assay (Fig. 4*C*), suggesting that Aurora-A forms a complex with activated RalA. In contrast, NIH3T3 cells were cotransfected with FLAG-tagged Aurora-A and HA-tagged RalA-S194A and immunoprecipitated using GST-RalBP1-RBD. The result showed that Aurora-A failed to form a complex with RalA-S194A (Fig. 4*C*). These findings suggest that the phosphorylation of RalA by Aurora-A may modulate the RalA function at the level of RalA activation and complex assembly.

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 (^{189}KKKRKSLAKRI^{199}) was synthesized and tested for serving as a substrate for recombinant Aurora-A/B. An *in vitro* peptide kinase assay indicated that Aurora-A, but not Aurora-B, efficiently phosphorylated RalA-Ser^{194} peptide with an apparent K_m 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. 5*A*), 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-Ser^{194}, ^{189}KKKRKSLAKRI^{199}, 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 [γ-^{32}P]ATP. Substituting Ala for Ser at the center (Ser^{194}) of the ^{189}KKKRKSLAKRI^{199} 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 Arg^{192} and Leu^{195} with Ala individually completely eliminated Aurora-A phosphorylation (Fig. 5*B*). 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 R*X*SL is the substrate specificity determinant for Aurora-A in ^{189}KKKRKSLAKRI^{199} 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-A/RalA-(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 had 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. 6*A*). 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



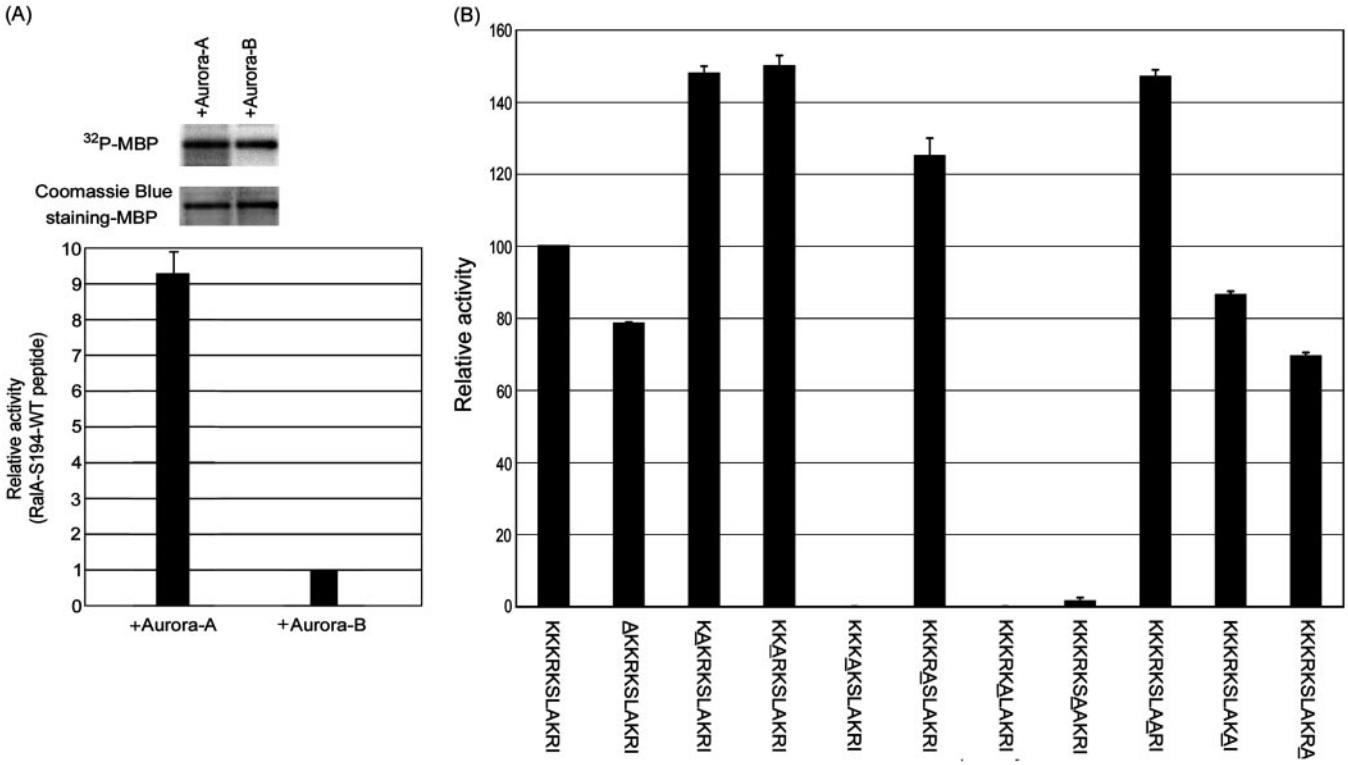


FIG. 4. **Aurora-A phosphorylates RalA on Ser^{194} and forms complex with activated RalA.** *A*, the sequence alignment between *RalA* and *RalB*. Three possible phosphorylation sites (Ser^{138}, Ser^{183}, and Ser^{194}) on RalA are indicated. *B*, recombinant GST-tagged RalA-WT, RalA-S138A, RalA-S183A, RalA-S194A, and RalB-WT were incubated with recombinant Aurora-A, respectively, in the presence of [γ-^{32}P]ATP. The samples were run on SDS-PAGE and transferred to a PVDF membrane followed by autoradiography (*top*) or Western blot analysis with anti-GST antibody (*bottom*). *C*, the HA-RalA-WT or HA-RalA-S194A construct was either expressed alone or coexpressed with indicated FLAG-tagged constructs (vehicle or FLAG-Aurora-A-WT) in NIH3T3 cells. Forty-eight hours after transfection, cells were lysed and immunoprecipitated with GST-RalBP1-RBD as described under "Experimental Procedures." Immunocomplexes were separated by SDS-PAGE and analyzed by Western blotting with anti-FLAG (*top*) and anti-HA antibody (*bottom*).

FIG. 5. **Characterization of the substrate specificity determinants in RalA-S194 peptide.** *A*, recombinant Aurora-A and Aurora-B were incubated with 2.5 μg myelin basic protein (*MBP*), respectively, as an exogenous substrate in the presence of [γ-^{32}P]ATP to ensure that the input of Aurora-A kinase activity was equivalent to that of Aurora-B (*top*). 5 μg of RalA-S194-WT peptide (*bottom*) was incubated with Aurora-A or Aurora-B, respectively, in the kinase reaction buffer with the presence of [γ-^{32}P]ATP. These reaction mixtures were spotted on P81 paper, washed, and counted as described under "Experimental Procedures." *B*, RalA-S194-WT (^{189}KKKRKSLAKRI^{199}) and its alanine mutant peptides were incubated with Aurora-A in the kinase reaction buffer with the presence of [γ-^{32}P]ATP. These reaction mixtures were spotted on P81 paper, washed, and counted as described under "Experimental Procedures."

and HA-tagged V23RalA-WT/S194A were verified via Western blotting (Fig. 6*B*), 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, 5×10^3 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 as well as all of the stable clones established in this study (data not shown). However, if collagen I was coated on the bottom side of the Transwell insert, an increase in cell motility was apparent in Aurora-A-WT-expressing MDCK cells, whereas both MDCK parental cells and Au-

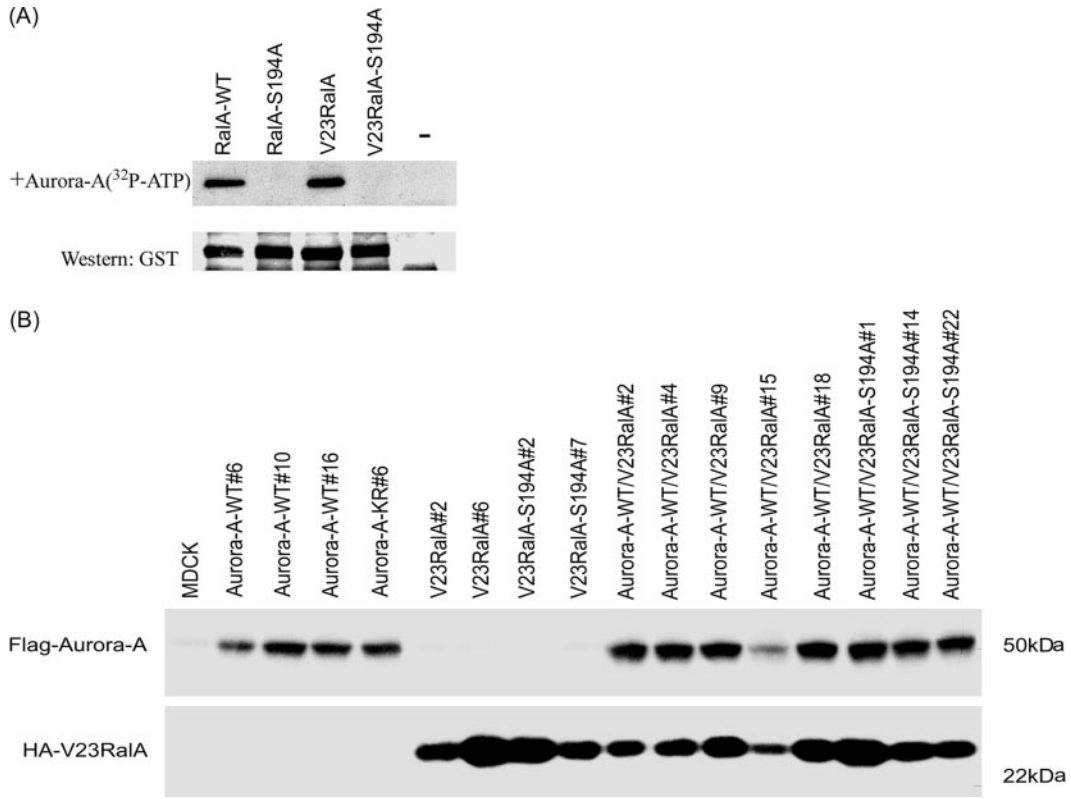


FIG. 6. **Establishment of Aurora-A/V23RalA stable clones in MDCK.** *A*, recombinant GST-tagged RalA-WT, RalA-S194A, V23RalA, and V23RalA-S194A were incubated with recombinant Aurora-A, respectively, in the presence of [γ-^{32}P]ATP. The samples were run on SDS-PAGE and transferred to a PVDF membrane followed by autoradiography (*top*) or Western blot analysis with anti-GST antibody (*bottom*). *B*, equal amounts of cell lysates (50 μg), prepared from MDCK (vector control) or various combinations of FLAG-Aurora-A-WT/KR and HA-V23RalA-(WT/S194A) MDCK stable clones, were immunoblotting with anti-FLAG or anti-HA antibody. The molecular markers (kDa) are shown on the *right*.

rora-A-KR-expressing MDCK cells had basal motility capacity (Fig. 7*A*). Fig. 7*B* 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. 7*B*). 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 Ser^{194} 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. 10^4 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. 8*A*, 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-Ser^{194}, 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. 8*B* 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*), compared with Aurora-A-WT alone. On the other hand, coexpression of V23RalA-S194A with Aurora-A-WT significantly abolished the colony formation (Fig. 8, *A* and *B*), implying that V23RalA-Ser^{194}, 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-Ser^{194} 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

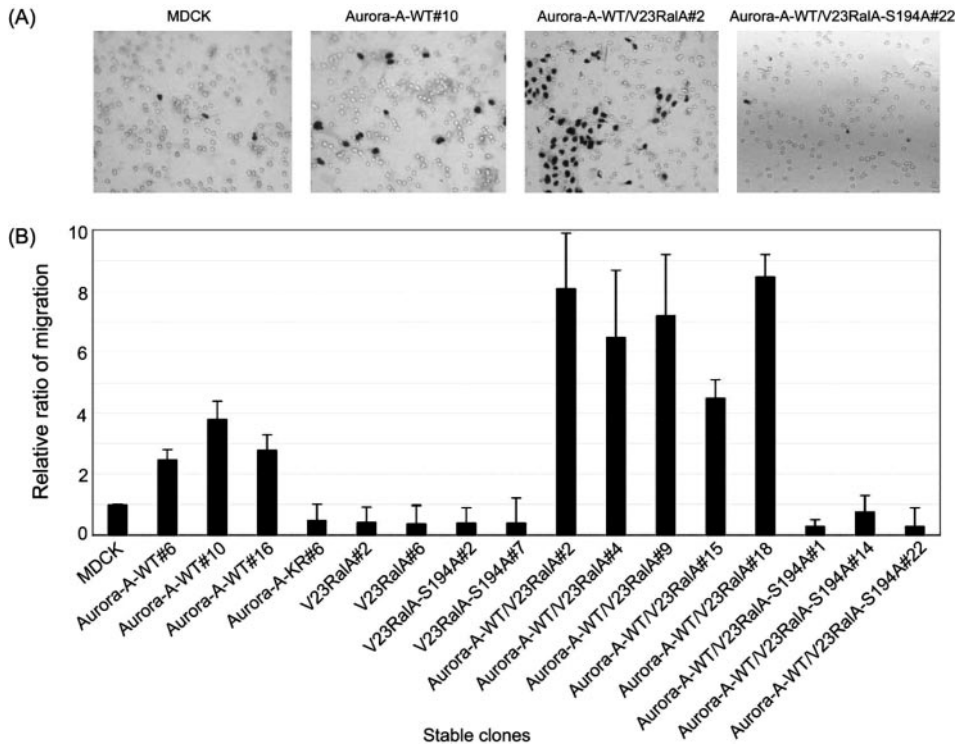


FIG. 7. **Effects of Aurora-A/V23RalA MDCK stable clones in collagen I induced cell migration.** *A*, 5×10^3 cells of MDCK (vector control) or various MDCK stable clones were seeded into the top of a Transwell insert, where collagen I was coated to the bottom of the dish. 22 h later, the cells on the 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.

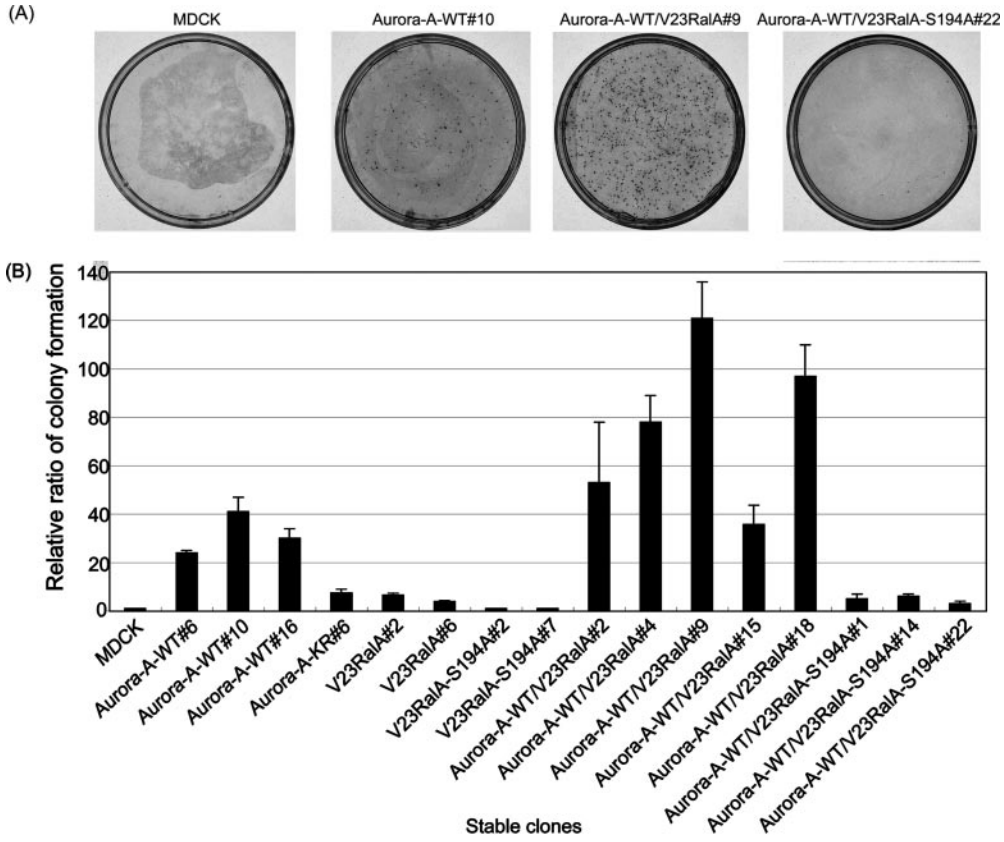


FIG. 8. **Effects of Aurora-A/V23RalA MDCK stable clones on anchorage independent cell growth ability.** *A*, 1×10^4 cells of MDCK (vector control) or various stable clones were plated in soft agar incubated in 37 °C, 5% CO_2/95% air environment incubator for 28 days. Subsequently, the colonies formed 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 oncogenic in murine NIH3T3 or Rat1 cells (6, 7), although no oncogenic 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 Ser^{194} and the substrate specificity determinant of RalA-S194 peptide is R*X*SL; and (*d*) Aurora-A stable clones in MDCK promote collagen I-induced cell motility and anchorage-independent growth ability through phosphorylation on V23RalA-Ser^{194}. 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 R*X*SL 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), Ral-GDS, osteopontin, and FLJ10877 fis, for which the phosphorylation motif has not yet been determined, contain the R*X*SL 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 require 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 hepatocyte 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-Ser^{194} 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 subsequently 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-Ser^{194} phosphorylation.

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