Phosphorylation of RalB Is Important for Bladder Cancer Cell Growth and Metastasis

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Abstract

RalA and RalB are monomeric G proteins that are 83% identical in amino acid sequence but have paralogue-specific effects on cell proliferation, metastasis, and apoptosis. Using in vitro kinase assays and phosphosite-specific antibodies, here we show phosphorylation of RalB by protein kinase C (PKC) and RalA by protein kinase A. We used mass spectrometry and site-directed mutagenesis to identify S198 as the primary PKC phosphorylation site in RalB. Phorbol ester [phorbol 12-myristate 13-acetate (PMA)] treatment of human bladder carcinoma cells induced S198 phosphorylation of stably expressed FLAG-RalB as well as endogenous RalB. PMA treatment caused RalB translocation from the plasma membrane to perinuclear regions in a S198 phosphorylation–dependent manner. Using RNA interference depletion of RalB followed by rescue with wild-type RalB or RalB(S198A) as well as overexpression of wild-type RalB or RalB(S198A) with and without PMA stimulation, we show that phosphorylation of RalB at S198 is necessary for actin cytoskeletal organization, anchorage-independent growth, cell migration, and experimental lung metastasis of T24 or UMUC3 human bladder cancer cells. In addition, UMUC3 cells transfected with a constitutively active RalB(G23V) exhibited enhanced subcutaneous tumor growth, whereas those transfected with phospho-deficient RalB(G23V-S198A) were indistinguishable from control cells. Our data show that RalA and RalB are phosphorylated by different kinases, and RalB phosphorylation is necessary for in vitro cellular functions and in vivo tumor growth and metastasis. Cancer Res; 70(21); OF1–10. ©2010 AACR.

Introduction

The Ras (Ras-like) proteins in the superfamily of small GTPases are the closest relatives of Ras, with 55% amino acid sequence identity (1). The two Ras genes, RalA and RalB, are expressed in human tissues and produce proteins that are >80% identical in sequence. Ras proteins are downstream effectors of Ras and contribute to Ras-induced transformation (2–6). Ras proteins have also been implicated in the regulation of endocytosis, exocytosis, actin cytoskeletal dynamics, and gene transcription (5, 7, 8). In addition, RalA and RalB contribute to cancer cell migration, invasion, and metastasis (9–14).

Ral GTPTases have both overlapping and distinctive roles in these cellular processes. Studies using RNA interference (RNAi) revealed that depletion of RalA severely impaired the anchorage-independent proliferation of cancer cell lines, whereas RalB was found to be essential for survival of a variety of tumor cell lines (15, 16). Studies using geranylgeranyltransferase I inhibitors (GGTI-2417) showed expression of GGTI-resistant farnesylated RalB, but not farnesylated RalA, conferred resistance to the proapoptotic and anti-anchorage-dependent growth effect of GGTI-2417. Conversely, farnesylated RalA, but not farnesylated RalB, rescued anchorage-independent cell proliferation in MiaPaCa2 cells (17). Knockdown of RalA in pancreatic cancer cells impaired tumor formation (12), whereas knockdown of RalB in prostate cancer cells inhibited bone metastasis (11). RalB depletion reduced bone metastasis in DU145 but not PC3 cells (11), whereas it inhibited metastasis by pancreatic cells, suggesting the cellular context may be important in determining the role of Ral paralogues in cancer. In bladder cancer, RalB but not RalA depletion by RNAi inhibited migration, whereas combined knockdown of both RalA and RalB inhibited growth (10). In vesicle transport, active RalA but not RalB was found to promote the assembly of the exocyst and enhance polarized delivery of membrane proteins (18). Together, these findings show that, despite extensive sequence identity, these Ras proteins have different cellular functions. Hence, we anticipate that Ral paralogues respond to different signals and/or engage different downstream effectors, but this has not yet been shown.

Pathways that activate Ral GTPTases include the GDP/GTP cycle regulated by guanine nucleotide exchange factors (GEF) and elevation of intracellular Ca²⁺ levels leading to interaction
of Ral GTPases with calmodulin (7). Recently, phosphorylation of RalA was found to regulate its activity in cell transformation (19–21). RalA was found as a phosphoprotein by searching for Aurora-A kinase substrates using small pool expression screening. Aurora-A kinase phosphorylates RalA at S194, increasing RalA activity and translocating RalA (19, 21). Proteomic analyses found RalB in complex with the Aβ subunit of protein phosphatase 2A (PP2A). PP2A dephosphorylates RalA at S183 and S194 and inactivates the protein (20). Currently, nothing is known regarding RalB phosphorylation, but the S183 and S194 sites in RalB are not conserved in RalB. These data led us to hypothesize that phosphorylation may be paralogue specific and that different kinases may regulate the Ral paralogues.

Here, we test this hypothesis and show that RalB is phosphorylated at S198 by protein kinase C (PKC). RalA and RalB are phosphorylated differentially by protein kinase A (PKA) and PKC. The S198 phosphorylation site is necessary for the effects of RalB on anchorage-independent growth and cell motility in vitro and the development of lung metastasis in vivo. Furthermore, S198 is necessary for manifestation of the growth promoting effect of GTP loaded RalB on in vivo tumor growth. Together, these data suggest RalB phosphorylation is an independent mechanism of regulating the biological activity of RalB.

Materials and Methods

Biochemical reagents and DNA constructs

Anti-RalB antibodies were from R&D Systems and Millipore. Monoclonal anti-FLAG M2 antibody, EZView Red Anti-FLAG M2 Affinity Gel, and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich. PKC kinase inhibitor Go-6983, calyculin-A, and anti-α-tubulin monoclonal antibody were from Calbiochem. Anti-green fluorescent protein (GFP) antibody was from Santa Cruz. [γ-32P]ATP was purchased from Perkin-Elmer Life and Analytical Sciences. NH2 terminal tagged glutathione S-transferase (GST)–RalA or GST-RalB fusion proteins were expressed in Escherichia coli strain BL21 (DE3) and purified by GST-Bind kits (Novagen) according to the manufacturer's instructions. PKA was purified to homogeneity from bovine heart (22). PKC was purified by sequential chromatography as multiple PKC isoforms from human RBC (23). Recombinant human Aurora-A kinase was expressed in bacteria and purified on Ni-NTA agarose (24). Plasmids encoding full-length human RalA and RalB were constructed in pCMV4-FLAG vector (Sigma-Aldrich) providing an NH2 terminal FLAG tag. GFP-RalB was constructed in pEGFP-C1 vector (Clontech). G23V mutants for active RalB or phospho mutants, RalB(S198A) and RalB(G23V-S198A), phosphomimetic RalB(S198D), were generated by QuickChange site-directed mutagenesis (Stratagene).

Generation of phospho-specific RalB antibody

Phosphospecific RalB antibody was generated by AbboMax, Inc. Briefly, two rabbits were immunized with KHL-conjugated RalB peptide centered on phospho-S198. The antisera were adsorbed against immobilized nonphosphopeptide, and the phospho-specific antibody affinity was purified with phosphopeptide.

Cells, transfection, immunoprecipitation, and Western blot

UMUC3, RT112, and T24 human bladder cancer cells and HEK293T cells were obtained from American Type Culture Collection and cultured as described (http://www.atcc.org/). The cells were transfected using FuGene 6 (Roche) according to the manufacturer’s instructions. T24 cells were transfected using Lipofectin (Invitrogen). UMUC3 cells or T24 cells stably expressing wild-type FLAG-Ral or mutant Ral or GFP-Ral or mutant GFP-Ral were selected in medium containing G418. Surviving cells were pooled and exogenous Ral protein expression was analyzed by Western blot. Surviving GFP-transfected cells were pooled and sorted using fluorescence-activated cell sorting (FACS; Flow Cytometry Core Facility, University of Virginia) to get stable cells with equal expression. For immunoprecipitation, the extracts were incubated with anti-FLAG M2 agarose or specific antibodies with beads for 2 hours at 4°C. The beads were washed and then either used as substrates for kinase assay or subjected to SDS-PAGE for Western blot.

Lentiviral short hairpin RNA (shRNA) expression vectors targeting RalB were purchased from Sigma, and UMUC3 stable RalB knockdown cells were established as manufacturer's instructions. RalB depleted cells were transfected with shRNA-resistant FLAG-RalB or mutant and selected in the medium containing G418. Surviving cells were pooled and analyzed by Western blot.

In vitro kinase assay

GST-RalA or GST-RalB fusion proteins or immunoprecipitates of FLAG-RalA, FLAG-RalB, or the mutants were incubated with purified kinases in kinase reaction buffer with [γ-32P]ATP for 30 minutes at 30°C. The products were analyzed by SDS-PAGE and autoradiography. Phosphorylation was quantified by excising the bands corresponding to the proteins and measuring radioactivity with a Beckman Model LS6500 scintillation counter.

Cell fractionation

RT112 cells were treated with or without 100 ng/mL PMA and 20 nmol/L calyculin-A for 10 minutes after overnight serum starvation. Cell membranes were prepared with ProteoExtract Native Protein Extraction kit (Calbiochem) following the manufacturer's instructions. Protein concentrations were determined by BCA protein assay (PIERCE).

Immunofluorescence

For immunofluorescence, the cells were plated on the cover glasses. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. The cells were stained with phalloidin-Alexa Fluor 594 (Molecular Probes) to visualize actin filaments and 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Images were acquired using Zeiss LSM 510 Meta confocal microscope.

Wound assay and Transwell migration assay

Wound assay was performed as previously described (25). T24 stable cells (2 × 104) were plated in a six-well plate in triplicate and incubated for 2 days. The cells were serum starved in HyQ-CCM1 medium for 24 hours, scraped, and
assayed with or without PMA. The assay was terminated when the wound of PMA-stimulated FLAG vector cells was almost closed. The wound was quantified using ImageJ. Cell migration was performed as described (10). UMUC3 stable cells (2 × 10^4) were seeded to the upper chamber of Transwell filters (8.0-μm pores, Becton Dickinson) in serum-free media in triplicate in a 24-well tissue culture plate. The lower chambers were filled with media supplemented with 2% fetal bovine serum (FBS). Cells that migrated through the chambers were stained with crystal violet after 6 hours of migration and counted.

**Anchorage-independent growth**

Cells (2 × 10^4) were suspended in 3-mL MEM medium containing 2% FBS and 0.4% SeaPlaque low melting temperature agarose (Cambrex) with or without PMA. The suspension was added onto a layer of MEM containing 1% SeaPlaque agarose in triplicate in a six-well plate. Plates were incubated at 37°C, and medium with or without stimulators replaced every 3 to 4 days. Colony numbers were quantified after 3 weeks using ImagePro 4.5 software.

**In vivo xenograft experiments**

Four-week-old athymic nude female mice were obtained from the National Cancer Institute breeding facility. For subcutaneous tumorigenicity, 1 × 10^6 of UMUC3 stable cells were injected s.c. into right and left flanks of mice. Five mice were injected for each cell line. Subcutaneous tumors were measured by calipers weekly, and tumor volume was calculated. To evaluate the ability of development of experimental lung metastases of UMUC3 cells, 1 × 10^6 of UMUC3 stable cells were injected into mice by tail vein. Ten mice were injected for each cell line. The detectable lung lesions were evaluated by Xenogen bioluminescent imaging (Caliper Life Sciences, Hopkinton; ref. 26). For the overall survival calculation, a mouse was counted if it died spontaneously or if any triggers to euthanasia were encountered, such as weight loss >20%, skin ulceration, signs of chronic pain, or distress. Euthanasia was carried out according to AVMA Guidelines on Euthanasia (Rev. June 2007).

**Statistical analysis**

Student’s t tests were used for comparisons in cell migration and anchorage-independent cell growth assays. Repeated measures models were used to compare tumor growth among groups. The models allowed the variability in tumor volumes to increase with time and F tests were used for pair-wise comparisons of groups and for comparisons over specific time points. For pooled analyses, tumor growth across multiple separate and independent experiments was analyzed using repeated measures models that allowed rates of tumor growth to differ across experiments. Significance was considered at P < 0.05. Analyses were carried out in SAS version 9.1, PROC MIXED. Kaplan-Meier curves were used to estimate overall survival in the metastasis experiment in vivo. The log-rank test was used to compare groups.

**Results**

**Raβ is phosphorylated at S198 by PKC**

The phosphorylation of Raβ by Aurora-A has been reported, but the corresponding region of Raβ has a different sequence, computationally predicted to be a PKC substrate. Thus, we used in vitro kinase assays to compare the phosphorylation of Raβ and Raβ by PKC, Aurora-A kinase, and PKA. Purified recombinant GST-Raβ and GST-Raβ fusion proteins were incubated with purified kinases plus [γ-32P]ATP, and incorporated radioactivity was visualized by autoradiography and quantified by scintillation counting. GST-Raβ was phosphorylated more effectively by PKC compared with either PKA or Aurora-A (Supplementary Fig. S1A, left). In contrast, under the same conditions, GST-Raβ was phosphorylated much more extensively by PKA than by either PKC or Aurora-A kinase (Supplementary Fig. S1A, right). Furthermore, FLAG-Raβ and FLAG-Raβ immunoprecipitated from 293T cells were also preferentially phosphorylated in vitro by different kinases. FLAG-Raβ was phosphorylated by PKC, but not by either PKA or Aurora-A, whereas FLAG-Raβ was phosphorylated to the same extent as FLAG-Raβ, but by PKA and not by either PKC or Aurora-A (Supplementary Fig. S1B).

To identify phosphorylation sites in FLAG-Raβ, we used mass spectrometry and mutagenesis. Mass spectrometry did not reveal any phosphorylated tryptic peptides, but the analysis did not cover the COOH terminal region from residue 175 to 206 (Supplementary Fig. S2). In the COOH terminal region of Raβ residue S198 corresponds to the position of S194 in Raα, which has been reported as a site of phosphorylation (19–21). We introduced a S198A mutation that reduced in vitro FLAG-Raβ phosphorylation by PKC ~70%, relative to wild-type Raβ as a control (Fig. 1A). These results indicated S198 is the primary but not the only PKC phosphorylation site(s) in Raβ. Other sites in the COOH terminal region of FLAG-Raβ reactive with PKC were not identified; however, we at least excluded S182 and S192 as potential sites of phosphorylation, which were predicted by algorithms in NetPhospho 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). Double mutants S198A-S182A and S198A-S192A and a triple mutant S198A-S182A-S192A were [32P]phosphorylated by PKC to the same extent as single mutant Raβ (S198A) (data not shown).

**Raβ is phosphorylated at S198 by endogenous PKC in living cells**

We transiently expressed FLAG-Raβ in 293T cells (Fig. 1B) or UMUC3 human bladder cancer cells (Fig. 1C) and treated the cells with PMA to activate endogenous PKC with or without pretreatment with PKC kinase inhibitor Go6983. Raβ phosphorylation was assayed by immunoblotting with a phosphosite-specific Raβ(pS198) antibody we developed. There was phosphorylation of S198 in FLAG-Raβ in 293T cells that was increased by PMA addition to the cells. Pretreatment of the cells with Go6983 effectively blocked PMA-induced phosphorylation of S198 in Raβ (Fig. 1B). Mutation of S198 in FLAG-Raβ eliminated phosphorylation of this site,
as expected, and no immunoblotting with the phosphosite antibody was seen with S198A RalB or the G23V-S198A activated RalB even in cells treated with PMA (Fig. 1B). Equivalent expression of the RalB proteins is shown by immunoblotting for FLAG. To generalize these observations, we evaluated UMUC3 human bladder cancer cells. In these cells, phosphorylation of S198 in FLAG-RalB was observed with PMA stimulation, and this response was effectively blocked by pretreatment with Go6983 (Fig. 1C). To show phosphorylation of endogenous RalB, we used RT112 human bladder cancer cells that have abundant RalB. Cells were treated with or without PMA to activate PKC and RalB was immunoprecipitated from a membrane fraction with anti-RalB antibody. Endogenous RalB phosphorylation in response to PMA was observed by immunoblotting with RalB(pS198) antibody (Fig. 1D). Taken together, these results show PKC phosphorylation of RalB at S198 in different cell lines.

**Phosphorylation of S198 in RalB affects RalB intracellular localization**

Given that RalA phosphorylation affects its localization (21), we used fluorescent microscopy to evaluate whether phosphorylation of S198 in RalB may also regulate cellular localization. For this, T24 cells stably expressing GFP-RalB or GFP-RalB(S198A) were serum starved overnight following -PMA/+PMA treatment. The cells were fixed, and images were acquired using Zeiss LSM 510 Meta confocal microscope. As shown in Figure 2A, phospho-RalB(S198) localization partially overlapped with that of GFP-RalB even in cells treated with +PMA. Immunoblot analysis of cell lysates confirmed the presence of S198 phosphorylation in both GFP-RalB and GFP-RalB(S198A) (Fig. 2B). These results suggest that phosphorylation of S198 in RalB may regulate its intracellular localization.
localization of this paralogue in T24 cells that we have studied before with regard to Ral function (25). We found that GFP-RalB was retained in the plasma membrane of T24 cells, but PMA treatment translocated wild-type RalB from the plasma membrane to a perinuclear region, whereas phosphosite-mutated RalB(S198A) protein was not translocated in response to PMA (Fig. 2A). The protein expression levels of GFP-RalB wild-type or mutant RalB(S198A) were shown by anti-RalB and anti-GFP antibodies, and GFP was used as a control (Fig. 2B). The results suggest that PKC activation by PMA mediates relocalization of RalB, and this response depends on the phosphorylation of S198.

Phosphorylation of RalB at S198 and anchorage-independent growth of bladder cancer cells

We established UMUC3 cell lines that stably express wild-type FLAG-RalB or the phosphosite mutant RalB(S198A) (Fig. 3A). The phosphorylation of wild-type RalB but not the S198A mutant was detected with RalB(pS198) phosphosite antibody by immunoblotting anti-FLAG immunoprecipitates from these PMA-treated cells (Fig. 3A). We compared these UMUC3 cell lines for their ability to form colonies in an anchorage-independent growth assay and observed a doubling of the number of colonies in response to PMA stimulation in cells expressing wild-type RalB, a significant increase relative to controls treated with vehicle. In contrast, UMUC3 cell lines stably expressing phosphosite mutant RalB(S198A) yielded the same number of colonies as controls, irrespective of PMA treatment (Fig. 3B). Thus, responsiveness to PMA required S198 in RalB, consistent with PKC-dependent phosphorylation of this site contributing to anchorage-independent growth.

We also established UMUC3 cell lines stably knocked down for RalB by infection with lentivirus encoding RalB shRNA (Fig. 3C). As a test of RalB function, these knocked down cells were transfected with plasmids encoding shRNA-resistant forms of wild-type RalB or RalB(S198A). Immunoblotting with anti-RalB indicated the deletion of endogenous RalB and ectopic expressed RalB, with anti-α-tubulin used as loading control. D, effect on anchorage-independent growth of the indicated UMUC3 stable cell lines from C. Colony numbers relative to scramble control (Con) cells are expressed as mean ± SEM from triplicate plates of two experiments. P value is from Student’s t test.

Figure 3. Effects of RalB phosphorylation on anchorage-independent growth of UMUC3 cells. A, immunoblot analysis of UMUC3 stable cell lines expressing FLAG-RalB wild-type or mutant RalB(S198A) by anti-RalB for Ral protein expression or anti-α-tubulin immunoblot for loading control. The cells were treated with 100 ng/mL PMA for 15 min. The FLAG-RalB and FLAG-RalB(S198A) were immunoprecipitated, and the phosphorylation of RalB was analyzed with anti-phospho-RalB(pS198) antibody. Immunoblotting with anti-FLAG was used as the control for recovery of RalB in immunoprecipitation. B, anchorage-independent growth of UMUC3 stable cell lines with and without PMA (0.05 nmol/L). Colony numbers relative to control cells with empty vector are expressed as mean ± SEM from triplicate plates of two experiments. P value is from Student’s t test. C, immunoblot analysis of RalB in UMUC3 stable cell lines, which were lentivirally infected with a scramble control (Con) or shRNA against RalB, complemented by an empty vector (vector), shRNA-resistant RalB or RalB(S198A) mutant. Immunoblot with anti-RalB indicated the deletion of endogenous RalB and ectopic expressed RalB, with anti-α-tubulin used as loading control. D, effect on anchorage-independent growth of the indicated UMUC3 stable cell lines from C. Colony numbers relative to scramble control (Con) cells are expressed as mean ± SEM from triplicate plates of two experiments. P value is from Student’s t test.

RalB Phosphorylation by PKC
Cancer Res; 70(21) November 1, 2010
found that RalB depletion significantly impaired anchorage-independent growth, reducing the number of colonies by a third (Fig. 3D). This phenotype was reversed by ectopic expression of wild-type RalB protein; however, the phosphosite mutant RalB(S198A) did not restore anchorage-independent growth (Fig. 3D). These data show that S198 in RalB is required for anchorage-independent growth of human bladder cancer cells. We concluded that phosphorylation of S198 modulates RalB function.

RalB S198 is required for cell motility and actin cytoskeletal organization

We have previously shown that the knockdown of RalB produced a reduction in actin stress fibers and a marked increase in filopodia extensions (10) and have recapitulated these findings here (Fig. 4A). Interestingly, expression of the shRNA-resistant wild-type RalB in knockdown cells restored the actin stress fibers and reduced the prominent filopodia, but expression of the RalB(S198A) mutant did not affect either of these phenotypes (Fig. 4A and B). In addition to effects on the cytoskeleton, RalB knockdown with lentivirus encoding RalB shRNA impaired Transwell cell migration in UMUC3 cells (Fig. 4B), and this paralleled the effects on the actin cytoskeleton (Fig. 4A and B). This phenotype was partially rescued by reexpression of wild-type RalB but not by RalB(S198A). There was a statistically significant difference in the rescue by wild-type versus S198A RalB.

We previously showed that PMA treatment stimulates cell motility in T24 human bladder tumor cells (25). We established T24 stable cells expressing either (a) FLAG-RalB wild-type, (b) nonphosphorylatable FLAG-RalB S198A, or (c) a phosphomimetic mutant FLAG-RalB S198D. In the wound migration assay, chemokinesis of these cells was stimulated by PMA, resulting in wound closure of the control T24 cells and T24 cells expressing wild-type FLAG-RalB protein (Fig. 4C). However, in the same time period, T24 cells expressing RalB(S198A) migrated much less, leaving most of the wound space open. Conversely, wounds were almost

**Figure 4.** Effects of RalB phosphorylation on the actin cytoskeleton and cell migration. A, effects on actin cytoskeleton. The indicated UMUC3 stable cells as described in Fig. 3C were fixed and stained with phalloidin-AlexaFluor 594 (red) to visualize actin filaments and DAPI (blue) to visualize nuclei. Arrows indicate filopodial extensions. B, Transwell migration and cell actin. Transwell migration was carried out with the indicated UMUC3 stable cell lines as described in Fig. 3C. Light gray columns, numbers of cells that migrated on the opposite side of the filter ("Migrated Cells," *) relative to scramble control cells (Con) set at 100 are expressed as mean ± SEM from triplicate plates. *, P < 0.001. P value is from Student’s t test relative to Con. Dark gray columns, the percentage of cells with actin stress fiber relative to untransfected cells (Δ) as a function of transfected construct. Most cells with shRNA RalB had actin reduction/absence and were used as "actin negative" in the scoring of other mutants. Typical images are shown in Fig. 4A. *, P < 0.001. P value is from Student’s t test relative to Con. C, wound assay. The assay was carried out with T24 human bladder carcinoma cells stably expressing FLAG-RalB or mutants RalB(S198A) and RalB(S198D). FLAG vector was used as control as described in Materials and Methods. Representative of the fields used to quantitate motility using ImageJ (top) and generate the graph (bottom). The average distances (microns) of wounds were shown as mean ± SEM from multifields of two experiments. *, P < 0.001.
completely closed by T24 cells expressing RalB(S198D), even without PMA treatment (Fig. 4C). Data from multiple independent experiments were quantified to show the lack of response to PMA by cells expressing RalB(S198A) and the enhanced baseline and PMA-independent response of cells expressing RalB(S198D). Taken together, these data indicate S198 in RalB is involved in actin cytoskeletal organization and cell motility.

**RalB phosphorylation at S198 is required for promotion of human bladder cancer tumor growth by activated RalB**

We evaluated tumors resulting from s.c. inoculation of UMUC3 human bladder cancer cells stably expressing either activated GFP-RalB(G23V), phosphosite-mutated GFP-RalB(G23V-S198A), or GFP vector were injected s.c. into mice. The average tumor volumes (mm³) of each group (n = 5) as indicated were shown as mean ± SEM. Figure shown is one representative of three experiments. Data were analyzed using repeated measures models. (See text for pooled data analysis of all three experiments and Supplementary Table 1 for averages of all three experiments.) B, proportion of tumors of each group present at different time points out of total possible sites (n = 10). C, protein expression in subcutaneous tumors. The lysates of tumor tissues were subjected to SDS-PAGE and analyzed by anti-GFP or anti-RalB antibodies. D, UMUC3 stable cell lines as described in Fig. 3C, knockdown RalB complemented by an empty vector, and shRNA-resistant RalB or RalB(S198A) mutant were injected into mice by tail vein. The detectable metastasis was determined by Xenogen (inset shows typical example of mice with lung metastasis at 6 wk). Kaplan–Meier curves were used to estimate disease-specific overall survival ("death" surrogates in animals with Xenogen detectable metastasis). The log-rank test with adjustment for multiple comparisons was used to compare groups (n = 10).

**Figure 5.** RalB phosphorylation is required for subcutaneous growth and experimental lung colonization by UMUC3 cells. A, UMUC3 cell lines stably expressing activated GFP-RalB(G23V), phosphosite mutant GFP-RalB(G23V-S198A), or GFP vector were injected s.c. into mice. The average tumor volumes (mm³) of each group (n = 5) as indicated were shown as mean ± SEM. Figure shown is one representative of three experiments. Data were analyzed using repeated measures models. (See text for pooled data analysis of all three experiments and Supplementary Table 1 for averages of all three experiments.) B, proportion of tumors of each group present at different time points out of total possible sites (n = 10). C, protein expression in subcutaneous tumors. The lysates of tumor tissues were subjected to SDS-PAGE and analyzed by anti-GFP or anti-RalB antibodies. D, UMUC3 stable cell lines as described in Fig. 3C, knockdown RalB complemented by an empty vector, and shRNA-resistant RalB or RalB(S198A) mutant were injected into mice by tail vein. The detectable metastasis was determined by Xenogen (inset shows typical example of mice with lung metastasis at 6 wk). Kaplan–Meier curves were used to estimate disease-specific overall survival ("death" surrogates in animals with Xenogen detectable metastasis). The log-rank test with adjustment for multiple comparisons was used to compare groups (n = 10).
than animals injected with either GFP or GFP-RalB(G23V-S198A). Tumor growth in animals injected with GFP and GFP-RalB(G23V-S198A) were similar (Fig. 5A). Comparison of overall tumor burden at each time point revealed that control GFP cells and those expressing GFP-RalB(G23V-S198A) had lower tumor formation at earlier time points than those expressing GFP-RalB(G23V) \((P < 0.041; \text{Fig. 5B})\). Importantly, at the end of the experiment, we isolated tumors and found similar levels of GFP proteins in all tumors by immuno blotting with anti-GFP or anti-RalB antibody (Fig. 5C). Together, these observations indicate that enhancement of \textit{in vivo} tumor growth by activated RalB requires S198 phosphorylation.

**RalB S198 is required for the development of experimental lung metastasis by UMUC3 cells**

We evaluated the development of experimental lung metastasis resulting from UMUC3 cell lines stably knocked down for RalB, knockdown RalB complemented by an empty vector, shRNA-resistant RalB, or RalB(S198A) as described in Fig. 3C. Following injection of cells into the tail vein of mice, the metastases were detected and monitored weekly by bioluminescence using a Xenogen instrument \((26)\). Kaplan-Meier curves were used to estimate disease-specific and overall survival, and the log-rank test with Bonferroni correction for multiple comparisons was used to compare groups (10 mice in each; Fig. 5D; Supplementary Table S2). Once a bioluminescent signal was detected and shown to increase over two consecutive weeks, mice were considered to have a lung metastasis and were thus no longer disease free. Mice that exhibited surrogates of death as defined by our animal care and use committee were considered as dead of disease. Mice injected with UMUC3 cells that had RalB knockdown survived with no metastasis, whereas most mice injected with UMUC3 cells expressing control shRNA died with obvious metastases up to 9 weeks. Mice with UMUC3 cells knocked down for RalB but expressing a shRNA-resistant wild-type RalB protein developed metastases at a similar rate to mice expressing control shRNA \((P = 0.1278 \text{in Supplementary Table S2})\). In contrast, mice expressing the phosphosite mutant RalB(S198A) did not restore RalB-dependent metastasis (Fig. 5D; \(P = 1.00\) in Supplementary Table S2). Together, this indicates that phosphorylation at S198 of wild-type RalB is necessary for the development of experimental lung metastasis of human bladder cancer cells. Not surprisingly, disease-specific survival paralleled overall survival (Supplementary Table S2).

**Discussion**

This study reports phosphorylation of RalA and RalB by different protein kinases. RalB is phosphorylated at S198 by PKC, whereas RalA is phosphorylated at S194 by PKA. The S198 site is phosphorylated in stably expressed RalB and, more importantly, in endogenous RalB in living cells activated by phorbol ester. The S198 site (KKS\(^{Xxx}\)FK) in RalB conforms to a motif recognized by PKC [(R/K)(R/K)], and mutation of this Ser to Ala reduced PKC phosphorylation by \(\sim 70\%\), with the other 30% of phosphorylation presumably occurring at another site. Mutation of S182 and S192, in addition to S198, did not eliminate phosphorylation of RalB, and mass spectrometry did not locate other sites of phosphorylation up to residue 175 of RalB. Whereas the identity of secondary PKC phosphorylation site(s) in RalB remains unknown, S198 clearly is the primary PKC phosphorylation site with functional consequences. Similarly, RalA was shown to be phosphorylated at multiple sites; besides major S194 sites, S183 was phosphorylated in response to suppression of phosphatase PP2A Aβ subunit \((20)\).

The phosphorylation of S198 is important for anchorage-independent cell growth, cell motility, actin cytoskeletal organization, and \textit{in vivo} bladder cancer tumorigenicity driven by active RalB. We also evaluated the role of RalB phosphorylation in regulating experimental lung metastasis in bladder cancer by using loss of function experiments. Similar to what was noted in prostate cancer for bone metastasis for the DU145 cell line \((11)\), RalB depletion by RNAi reduced lung colonization and increased survival in UMUC3 cells. Importantly, when we restored Ral expression to endogenous levels by expressing wild-type RalB and phosphosite mutant RalB(S198A), only the former restored the metastatic competence of the cells. This indicates that phosphorylation of RalB is important for the development of lung metastasis in human bladder cancer cells.

Our finding that RalA and RalB are phosphorylated by different kinases may explain in part the different cellular actions of these paralogues, despite nearly identical protein sequence. The situation with RalA and RalB may be similar to the Ras family member Rap1, which is phosphorylated by PKA. Phosphorylation alters the ability of Rap1 to associate and regulate its downstream targets \((27–30)\). Previous reports claimed that phosphorylation of RalA at S194 by Aurora-A kinase regulates RalA activity as measured by effector RalBP1 binding and function in cells \((19, 21)\). We compared the binding of wild-type and S198A RalB with downstream effectors RalBP1, Sec5, and Exo84, but coprecipitation with these effectors was not different. The phosphorylation of RalB may affect binding to another yet undetermined protein in bladder cancer cells. Phosphorylation of RhoA by PKA induces relocalization of RhoA \((31, 32)\). Recently, it was reported that RalA phosphorylation at S194 by Aurora-A kinase translocated RalA from the plasma membrane \((21)\). Here, in human bladder cells, we observed phosphorylation of RalB by PKC induced RalB translocation from plasma membrane to perinuclear regions. This might be the functional consequence of RalB phosphorylation.

RalB phosphorylation by PKC has interesting implications for human bladder cancer. RalB has been implicated in bladder cancer migration and metastasis \((10)\), and PKC is known to play an important role in cellular differentiation and in malignancy and metastasis \((33–35)\). PKC activity is elevated in invasive human bladder carcinoma cell line EJ and inhibition of kinases by staurosporine inhibited invasion of this cell line \((36)\). PMA also upregulated vascular endothelial growth factor (VEGF) expression via PKC signaling in human bladder transitional carcinoma cell line RT4, and this was inhibited by staurosporine. VEGF is known as a key factor in...
human tumorigenesis and metastatic potential (37) and has been shown to be regulated by Ral GTPases (38). In colon carcinoma cells, PKCα contributes to high migratory activity (39), and a PKC inhibitor effectively inhibits migration and invasion of bladder carcinoma cells (40). Taken together, there seems to be substantial evidence for involvement of RalB and PKC in human bladder progression. Importantly, by linking PKC and RalB, our results may expose one of the underlying mechanisms whereby PMA and other PKC activators act as tumor promoters in cancer because RalB is involved in multiple cancer types (9–14). Based on these data, work to determine which PKC isoforms affect S198 in RalB seems warranted.

In summary, RalB phosphorylation translocates RalB from plasma membrane to perinuclear regions in an S198-dependent fashion and is necessary for RalB effects on cell migration and anchorage-independent growth in vitro and tumor growth and metastasis in vivo, irrespective of RalB activation by GTP loading. Importantly, these results suggest that RalB functions as an "AND" gate in signaling, wherein both GTP loading and S198 phosphorylation are required for full biological activity. We should look for circumstances where RalGEFs and PKCs are coincidently activated as conditions for RalB action. Because PKCs are implicated in bladder cancer progression and react with S198, inhibition of PKC, activation of the S198 phosphatase, or mimicking the phosphosite to interfere with RalB targeting could prove useful for treating metastatic bladder cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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