Tumorigenesis and Neoplastic Progression

Rap1GAP Inhibits Tumor Growth in Oropharyngeal Squamous Cell Carcinoma

Zhaocheng Zhang,* Raj S. Mitra,*
Bradley S. Henson,* Nabanita S. Datta,[†]
Laurie K. McCauley,^{†‡} Pawan Kumar,*
Julia S.-J. Lee,[§] Thomas E. Carey,^{*¶} and
Nisha J. D'Silva*

From the Departments of Oral Medicine, Pathology, and
Oncology,* and Periodontology, Prevention, and Geriatrics,[†]
University of Michigan School of Dentistry, Ann Arbor; and the
Departments of Pathology[‡] and Otolaryngology, Laboratory of
Head and Neck Cancer Biology,[§] and Biostatistics Core,[§] The
University of Michigan Medical School and the Comprehensive
Cancer Center, Ann Arbor, Michigan

Rap1, a growth regulatory protein that is strongly expressed in human squamous cell carcinoma (SCC), is inactivated by rap1GAP. Recent evidence in normal rat cells suggests that rap1GAP regulates proliferation. The objective of the current study was to investigate whether rap1GAP functions as a tumor suppressor in SCC. Using a pull-down assay, active GTP-bound rap1 was up-regulated in SCC compared to normal or immortalized keratinocytes. Because both rap1A and rap1B isoforms of rap1 are expressed in SCC, the rap1GAP inactivation of both rap1 isoforms was verified using cells transfected with EGFP-rap1A or EGFP-rap1B or co-transfected with FLAG-tagged rap1GAP. The results demonstrate that expression of rap1GAP in oropharyngeal SCC down-regulated active rap1, ERK activation, and proliferation. Incubation of stably transfected SCC cells with nocodazole, an inhibitor of mitosis, caused a slower accumulation of rap1GAP-transfected cells in the G₂ phase, in comparison to the vector control, indicating that rap1GAP-transfected cells have slower progression through the cell cycle. This was supported by down-regulation of cyclin D1, cdk4, and cdk6 in rap1GAP-transfected SCC cells. Furthermore, SCC cells transfected with rap1GAP produced significantly smaller tumors in nude mice as compared to controls ($P < 0.01$). These novel findings suggest that rap1GAP acts as a tumor suppressor protein in SCC. (Am J Pathol 2006; 168:585–596; DOI: 10.2353/ajpath.2006.050132)

Rap1A and rap1B are ras-like proteins that are ubiquitously expressed. Rap1A, which is also referred to as smg p21 or Krev1, shares 95% homology with rap1B, differing by only nine amino acids (of a total of 184), six of which are located at the C-terminus between amino acids 171 to 184.¹ Active rap1 modulates cellular functions by regulating the translocation of other proteins or by shuttling between intracellular compartments.^{1,2} Activation of rap1 occurs by switching between inactive guanine diphosphate (GDP)- and active GTP-bound forms. Rap1 activation is regulated by several guanine nucleotide exchange factors (GEFs) that include DOCK4, C3G, Epac, smgGDS, PDZ-GEFs, and CalDAG-GEF1.³ Mutations in these regulatory proteins have been identified in some malignancies. Osteosarcoma cells with dominant-negative DOCK4 do not form adherens junctions and are invasive.⁴ An activating mutation in CalDAG-GEF1, which would lead to an increase in active rap1, was identified in acute myelogenous leukemia.⁵ Rap1 is inactivated by GTPase-activating proteins (rap1GAPs) that induce GTPase activity.⁶ Loss of function or loss of expression of rap1GAP, thereby increasing GTP-bound rap1, induces cell proliferation. For example, the rap1GAP homologue tuberlin (TSC2) is mutated in tuberous sclerosis, which manifests with multiple benign tumors.⁷ Mutations in SPA-1, another rap1GAP homologue, affect the hematopoietic system.⁸ Recently, a tumor suppressor role was proposed for rap1GAP based on *in vitro* studies in rat thyroid epithelial cells.^{9,10} However, a tumor suppressive function for rap1GAP in cancer cells remains unexplored.

Active rap1 induces the mitogen-activated protein kinase (MAPK)-dependent signaling cascade,¹¹ which has been implicated in proliferation, differentiation, adhesion, and apoptosis.¹² Rap1A was originally identified as a ras-tumor suppressor gene in ras-transformed NIH 3T3 fibroblasts.¹³ In contrast, rap1A was subsequently shown

Supported by the National Institute of Dental and Craniofacial Research (grant DE00452-01 to N.J.D.) and the National Cancer Institute (SPORC grant P50 CA97248).

Accepted for publication October 19, 2005.

Address reprint requests to N. J. D'Silva, 1011 N. University Ave., Rm. 5217, Dept. of Oral Medicine, Pathology, and Oncology, University of Michigan, School of Dentistry, Ann Arbor, MI 48109-1078. E-mail: njdsilva@umich.edu.

to facilitate cell proliferation by inducing DNA synthesis when microinjected into Swiss 3T3 cells.¹⁴ Rap1B, the other rap1 isoform, has also been reported to induce proliferation and morphological transformation of Swiss 3T3 cells.¹⁵ These studies were performed by overexpression of one of the rap1 isoforms^{16,17} or by the effects of dominant-negative mutants. In rat thyroid cells and subsequently in the thyroid gland of a transgenic mouse, endogenous rap1B has been shown to have a mitogenic effect.^{18,19} However, little is known about the effects of inactivation of endogenous rap1 in malignant cells.

We recently showed that active rap1 is strongly expressed in human oral cancer,¹⁶ leading us to hypothesize that rap1GAP may be involved in tumor growth. In the present study, the effects of rap1GAP on tumor growth in human oropharyngeal squamous cell carcinoma (SCC) were investigated. Active rap1 was more strongly expressed in SCC cells than in normal or immortalized, nonmalignant keratinocytes. Furthermore, rap1GAP inhibited MAPK and blocked cell-cycle progression in SCC cells. Stable expression of rap1GAP in SCC inhibited proliferation *in vitro* and tumor growth *in vivo*.

Methods and Materials

Cell Culture

Oropharyngeal SCC cell lines²⁰ and HEK 293T cells were cultured as described previously in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml L-glutamine.¹⁶

Western Blot Analysis

Whole-cell lysates were prepared as described previously.¹⁶ Briefly, cell lines grown to 60 to 80% confluence were washed with ice-cold phosphate-buffered saline and lysed in 1% Nonidet P-40 lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl, 2 mmol/L MgCl₂, with protease inhibitors, and 10% glycerol). Particulate material in the lysates was pelleted by centrifugation, and the supernatant (Nonidet P-40 extract) was collected. Protein content was measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Proteins were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Nonspecific binding was blocked by incubation of membranes in Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20 (Bio-Rad). Membranes were incubated in the primary antibody for 1 hour at room temperature or overnight at 4°C. Primary antibody concentrations used were mouse anti-Flag monoclonal antibody M2 (Sigma Chemical Co., St. Louis, MO) 1:10,000 to 20,000; mouse anti-rap1 monoclonal antibody (Transduction Laboratories, Lexington, KY) 1:500 to 1000; goat anti-rap1GAP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) 1:2000 to

4000; rabbit anti-rap1GAPI and goat anti-rap1GAPII polyclonal antibodies (Santa Cruz Biotechnology), 1:200 and 1:500, respectively; rabbit anti-GFP antibody (Santa Cruz Biotechnology) 1:2000 to 4000; mouse anti-phosphorylated ERK1/2 (1:1000), rabbit anti-ERK1/2 (1:1000), rabbit anti-phosphorylated Akt (1:1000), and rabbit anti-Akt (1:1000, all from Cell Signaling, Beverly, MA); mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Chemicon Int., Temecula, CA), 1:10,000; mouse cyclin D1 (Santa Cruz Biotechnology) 1:1000 to 2000, and affinity-purified rabbit polyclonal anti-cyclin A, -cdk2, -cdk4, and -cdk6 antibodies (Santa Cruz Biotechnology), each at 1:2000. Affinity-purified secondary antibodies (donkey anti-rabbit IgG, goat anti-mouse IgG and donkey anti-goat IgG) conjugated with horseradish peroxidase (1:10,000 to 1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect primary antibodies. Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent system (Pierce, Rockford, IL) and exposed to X-ray film.

Rap1 Activation Assay

Rap1 activation was assayed by baiting rap1 with the rap1-binding domain of ral-GDS, which binds only the active GTP-bound form of rap1.²¹ The construct for ral-GDS was a generous gift from Dr. Johannes L. Bos (University Medical Centre Utrecht, Utrecht, The Netherlands). Glutathione S-transferase (GST)-tagged ral-GDS was purified from bacterial cells expressing the protein, linked to beads, and used to pull-down the active form of rap1, as described previously.^{16,21}

Transfections

The EGFP-rap1B construct was described previously.¹⁶ Using a similar strategy, a full-length polymerase chain reaction fragment of wild-type rap1A was subcloned from the pTARGET constructs, described previously,¹⁶ into the pEGFP-C1 vector (Clontech, Palo Alto, CA) at *Hind*III and *Eco*R1 sites. The newly synthesized plasmid, designated EGFP-rap1A, was sequenced to verify the presence and orientation of the rap1A cDNA.

Flag-tagged pcDNA 3.1-rap1GAP plasmid was a kind gift from Dr. P. Stork (Oregon Health Sciences University, Portland, OR). The pcDNA 3.1 empty vector was used as a control for transfection effects on endogenous gene expression. Catalytically inactive rap1GAP and control vector were obtained from Dr. J. Bos (Utrecht University Medical Center).²²

Transient Transfection

HEK 293T cells were seeded at 1×10^6 cells/60-mm dish and transfected with EGFP-rap1A or EGFP-rap1B with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At 24 hours after transfection, cells were harvested and whole-cell lysates were prepared as described above.

Stable Transfection

HEK 293T and UM-SCC-11A cells were transfected with pcDNA and pcDNA-Flag-rap1GAP plasmids. For HEK 293T cells, single clones were selected in the presence of hygromycin (500 $\mu\text{g/ml}$). For UM-SCC-11A, a mixed clonal population was selected in the presence of G418 (250 $\mu\text{g/ml}$) and used because we were unable to isolate multiple single clones.

In Vitro Proliferation Assays

For proliferation assays, stably transfected UM-SCC-11A ($\sim 2 \times 10^4$) or HEK 293T (5×10^4) cells were seeded into 60-mm dishes. The total number of cells was determined by trypan blue enumeration assays.

In Vivo Studies

For studies of rap1GAP on tumor growth *in vivo*, athymic nude mice (athymic Ncr nu/nu strain; NCI DTP), 18 to 25 g, were used. The mice were 4 to 6 weeks of age at the time of inoculation. After anesthesia, five mice were injected subcutaneously with 1×10^5 UM-SCC-11A cells stably transfected with rap1GAP or vector control. The cells were suspended in Dulbecco's modified Eagle's medium-fetal bovine serum/Matrigel (1:1; Becton Dickinson, Mountain View, CA). The animals were euthanized after 14 days, and tumor weight and volume were quantified. The presence of tumor was confirmed by hematoxylin and eosin staining of 5- μm tissue sections and visualization by light microscopy.

Immunohistochemistry

Tissue sections (5 μm) from formalin-fixed, paraffin-embedded tumor tissue were deparaffinized and rehydrated. Antigen retrieval was performed with 10 mmol/L sodium citrate buffer, pH 6, at 95°C. The sections were stained with affinity-purified mouse anti-cytokeratin 5/6 monoclonal antibody (DAKO, Carpinteria, CA) or anti-Flag antibody (Sigma). Immunodetection was performed as described previously.¹⁶

Phosphatase Assay

Whole-cell lysates (100 μg) were incubated with 20 U of calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) for 30 minutes at 30°C. Subsequently, 20 μg of this sample was immunoblotted with rap1GAP antibody.

Flow Cytometry

For flow cytometry, stably transfected UM-SCC-11A (3×10^5) or HEK 293T (3×10^4) cells were seeded into 60-mm dishes or six-well plates, respectively. Twenty-four to forty-eight hours after seeding, the cells were treated with 200 ng/ml (UM-SCC-11A) or 100 ng/ml (HEK 293T) of

nocodazole, an inhibitor of mitosis that arrests the cells at the G_2/M transition of the cell cycle. At different time points as indicated in the figure legends, cells were trypsinized, counted, and fixed in 70% ethanol for at least 1 hour at 4°C. Cells were subsequently resuspended in propidium iodide solution containing 0.1% sodium citrate, 25 $\mu\text{g/ml}$ propidium iodide (Sigma), 100 $\mu\text{g/ml}$ RNase A, 0.1% Triton X-100, and analyzed for DNA content by flow cytometry (flow cytometry core facility, University of Michigan Cancer Center) with BD Biosciences (San Jose, CA) FACSCalibur machine. Ten thousand cells were analyzed for each condition, and the proportion of cells in G_1 , S, and G_2/M phases was determined using Modfit cell cycle analysis software (Verity Software, Topsham, ME).

Data Analysis

Densitometric analysis was performed using AlphaEase software, version 5.5 (Alpha Innotech Corp., San Leandro, CA).

Statistical Analysis

For the *in vitro* studies, statistical analysis was performed using Student's *t*-test. For the *in vivo* studies, because both groups of stable cell lines (transfected with pcDNA or rap1GAP) were injected in the same mouse, univariate repeated measures analysis of variance was used to evaluate the differences in tumor volume and weight between cells transfected with empty vector and rap1GAP. Pairwise comparisons were evaluated through contrast method. Statistical programming was performed in SAS (Statistical Analysis System v8.2; SAS, Carey, NC). All tests were two-sided. A *P* value of 0.05 or less was considered statistically significant.

Results

Rap1GAP Inhibits Activation of Both Rap1A and Rap1B

Both rap1A and rap1B isoforms are expressed in normal and malignant keratinocytes (SCC).^{16,17} To investigate whether rap1GAP inhibits activation of both rap1A and rap1B, HEK 293T cells were co-transfected with wild-type EGFP-rap1A or EGFP-rap1B +/- rap1GAP. Rap1 isoform activation was assayed by pull-down assays using the rap-binding domain of ral-GDS, which binds only the active, GTP-bound form of rap1.²¹ The results for rap1A are shown on the left side of Figure 1, A–D, and for rap1B on the right side (Figure 1, E–H). In cells transfected with EGFP or EGFP-rap1A, the active GTP-bound form of endogenous (Figure 1A, EGFP) or endogenous and EGFP-rap1A (Figure 1A, EGFP-rap1A), respectively, were detected in ral-GDS precipitates. In contrast, in cells co-transfected with rap1GAP, active GTP-bound EGFP-rap1A and endogenous rap1GTP were undetectable (Figure 1A, EGFP-rap1A+GAP), consistent with in-

Figure 1. Rap1GAP inhibits activation of rap1A and rap1B. The HEK 293T cells were transiently transfected with EGFP (empty vector) or EGFP-tagged rap1A or rap1B (EGFP-rap1A and EGFP-rap1B, respectively), in the presence or absence of co-transfection with Flag-tagged Rap1GAP. Whole-cell lysates were isolated 24 hours after transfection. The active form of rap1 in each cell group (200 μ g each) was retrieved by ral-GDS pull-down (rap1-GTP, **A** and **E**), electrophoresed, transferred to nitrocellulose membranes, and blotted with rap1 antibody. To verify expression of the EGFP-tagged rap1 fusion proteins, whole-cell lysates (30 μ g each) were blotted with GFP (**B**, **F**) and rap1 antibodies (**C**, **G**), respectively. Flag-tagged rap1GAP co-expression was confirmed by Flag antibody (**D**, **H**).

activation of rap1. The presence of the transfected EGFP-rap1 and EGFP control were confirmed by antibody to EGFP in whole-cell lysates (Figure 1B). EGFP-rap1A migrates more slowly than EGFP alone due to the higher molecular mass of the fusion protein (Figure 1B). Antibody to rap1 shows that expression of total rap1 including endogenous rap1 and EGFP-rap1A (Figure 1C) was equivalent in the three transfection groups. Similar results were observed in HEK 293T cells transfected with EGFP alone, EGFP-rap1B, or EGFP-rap1B+rap1GAP (Figure 1, E–H). As with rap1A, co-transfection with rap1GAP decreased activation of EGFP-rap1B and abrogated activation of endogenous rap1 (Figure 1E, EGFP-rap1B+GAP). Although some GTP-bound rap1B was detected in the rap1GAP co-transfection, the expression of exogenous total EGFP-rap1B was much greater than that of endogenous total rap1 (Figure 1G) or the level of EGFP-rap1A (Figure 1A) that was fully inactivated by rap1GAP. Expression of EGFP and EGFP fusion proteins and of co-transfected FLAG-tagged rap1GAP were confirmed by immunoblotting with anti-GFP (Figure 1, B and F) and anti-FLAG (Figure 1, D and H) antibodies, respectively. Thus rap1GAP induces inactivation of both rap1A and rap1B.

Rap1GAP Slows Proliferation in HEK 293T Cells

Initial studies investigated the effects of rap1GAP on proliferation in HEK 293T, a transformed but nonmalignant human renal epithelial cell line that has a high transfection efficiency. Cells transfected with rap1GAP were placed under hygromycin selection for 3 weeks. Several

Figure 2. Selection of rap1GAP-expressing clones in HEK 293T cells. HEK 293T cells were stably transfected with pcDNA3.1 (empty vector) or pcDNA-Flag-rap1GAP. Single clones were selected in the presence of hygromycin. Whole-cell lysates from control (pcDNA) or rap1GAP (GAP1, GAP2, GAP3, GAP4) hygromycin-resistant clones were isolated. Lysates (60 μ g each) were electrophoresed, transferred to nitrocellulose, and blotted with Flag (**A**), rap1GAP (**B**), GAPDH (**C**), or rap1 antibodies (**D**, **E**). The active form of rap1 (rap1-GTP) in each cell group (600 μ g each) was retrieved by ral-GDS pull-down (**D**) and blotted with rap1 antibody. Cell lysates (**F**, 100 μ g) were incubated in the presence or absence of alkaline phosphatase and 20 μ g of this lysate was immunoblotted with rap1GAP antibody.

clones were isolated and screened for rap1GAP expression and function. The results from four representative clones (GAP1, 2, 3, 4; lanes 2 to 5) are shown in Figure 2. Both the FLAG (Figure 2A, lanes 2 to 5) and rap1GAP (Figure 2B, lanes 2 to 5) antibodies detected rap1GAP in all four clones. As expected, the antibodies gave no signal in the pcDNA control (empty vector) (Figure 2, A and B; lane 1). Equivalency of protein loading in all five lanes, including the pcDNA control lane, was verified by reprobing the filter from panel B with anti-GAPDH anti-

body (Figure 2C). Of the clones screened, differences in molecular mass of the FLAG-rap1GAP were apparent. Two clones, GAP2 and GAP4, exhibited three bands (Figure 2, A and B; lanes 3 and 5) whereas the other clones, GAP1 and GAP3, exhibited two bands (Figure 2, A and B; lanes 2 and 4).

Assessment of functional rap1GTP, performed with the ral-GDS pull-down assay, showed that rap1 activation was greatly reduced or abrogated in all four clones (Figure 2D, lanes 2 to 5). In contrast, active GTP-bound rap1 was strongly expressed in the pcDNA empty vector control cells (Figure 2D, lane 1). Equivalency of rap1 expression in the four different cell clones and pcDNA empty vector was verified by blotting whole-cell lysates with anti-rap1 antibody (Figure 2E). All five lysates expressed total rap1 equivalently, indicating that inactivation of GTP-bound rap1 (Figure 2D, lanes 2 to 5) is specific to expression of the exogenous rap1GAP.

Multiple peptide signals for rap1GAP have been reported previously in several different cells.^{10,23–26} Some of these differences in molecular mass of rap1GAP (Figure 2, A and B) are due to phosphorylation at multiple sites.²⁷ To assess the role of phosphorylation in protein migration, whole-cell lysates from each of the four clones were treated with phosphatase (Figure 2F). On phosphatase treatment, several of the bands were eliminated leaving two distinct signals (Figure 2F).

The effects of rap1GAP on proliferation were investigated in the stably transfected cells. As shown in Figure 3A, there was a significant growth delay observed in the four HEK 293T clones expressing pcDNA-FLAG-rap1GAP compared to cells stably transfected with the empty vector. This difference was significant as early as 3 days after seeding. To verify that the effects of rap1GAP on proliferation were due to inactivation of rap1, proliferation assays were performed on 293T cells transfected with inactive rap1GAP, a catalytically inactive mutant with RKR to LIG mutations in amino acids 284 to 286 rendering it unable to inactivate GTP-bound rap1.²² In contrast to functional rap1GAP, catalytically inactive rap1GAP (InGAP) did not exhibit inhibitory effects on proliferation (Figure 3B). To verify that catalytically inactive rap1GAP did not inactivate rap1A and rap1B, HEK 293T cells were co-transfected with wild-type EGFP-rap1A or EGFP-rap1B +/- inactive rap1GAP (Figure 3C). In cells transfected with rap1A alone or co-transfected with inactive rap1GAP, the active GTP-bound form of EGFP-rap1A and endogenous rap1 were detected (Figure 3C, left; EGFP-rap1A or EGFP-rap1A + InGAP). The presence of the transfected EGFP-rap1 and EGFP control were confirmed by immunoblotting whole-cell lysates with EGFP antibody (Figure 3C). Antibody to rap1 shows that expression of total rap1 including endogenous rap1 and EGFP-rap1A (Figure 3C) was equivalent in the three transfection groups. Similar results were observed in 293T cells transfected with EGFP alone, EGFP-rap1B, or EGFP-rap1B + inactive rap1GAP (Figure 3C, right; EGFP-rap1B or EGFP-rap1B + InGAP).

To investigate if rap1GAP's inhibitory effects on proliferation were due to effects on the cell cycle, we studied three of the HEK 293T clones stably expressing

Figure 3. Rap1GAP inhibits proliferation and slows cell-cycle progression of HEK 293T cells. **A:** Cell proliferation. The HEK 293T cells stably transfected with empty vector (pcDNA) or rap1GAP (GAP1, GAP2, GAP3, GAP4) were seeded at 50,000 cells per 60-mm dish, and proliferation was measured as described in the Materials and Methods section at days 3, 4, 5, and 6 after seeding. The values represent means of cell number + SD based on three wells/data point in a single experiment (* $P < 0.01$ versus the corresponding control). The experiment is representative of three experiments with similar results. **B:** Inactive rap1GAP does not inhibit cell proliferation. HEK 293T cells were transfected with empty vector or inactive rap1GAP (InGAP) and cell proliferation was determined at days 2, 3, and 4 after seeding. **C:** Catalytically inactive rap1GAP does not inactivate rap1A or rap1B. The HEK 293T cells were transiently transfected with EGFP (empty vector) or EGFP-tagged rap1A or rap1B (EGFP-1A and EGFP-1B, respectively), in the presence or absence of co-transfection with inactive Rap1GAP (InGAP). Twenty-four hours after transfection, whole-cell lysates were isolated. The active form of rap1 in each cell group was retrieved by ral-GDS pull-down (rap1-GTP), electrophoresed, transferred to nitrocellulose membranes, and blotted with rap1 antibody. To verify expression of the EGFP-tagged rap1 fusion proteins, whole-cell lysates (30 μ g each) were blotted with GFP and rap1 antibodies, respectively. Inactive rap1GAP co-expression was confirmed by rap1GAP antibody.

Figure 4. Cell-cycle analysis by flow cytometry. The HEK 293T cells stably transfected with empty vector (pcDNA) or rap1GAP (three clones: GAP2, GAP3, GAP4) were seeded at 30,000 cells per 60-mm dish. After 48 hours, cells were incubated with 100 ng/ml of nocodazole. **A–D:** Cells were collected at 0, 6, 10, and 16 hours after nocodazole treatment. Cell-cycle analysis was performed as described in the Materials and Methods section. The data are representative of duplicate experiments. **E:** Percentage of cells in the G₂ phase of the cell cycle at 0, 6, 10, and 16 hours after nocodazole treatment (summarized from **A–D**).

rap1GAP. Vector control and rap1GAP cells exhibited similar cell-cycle profiles in the absence of nocodazole treatment (Figure 4, A and E). However, at 10 hours after treatment with nocodazole, which arrests cells in the G₂ phase, clones expressing rap1GAP had a greater proportion of cells in the S phase and a smaller proportion of cells in the G₂ phase than the vector control (Figure 4, C and E). In fact, the vector control exhibited a higher G₂ phase than rap1GAP-transfected cells at 6 hours after nocodazole treatment (Figure 4, B and E). However, by 16 hours after nocodazole treatment, the cell-cycle distribution was again similar between control vector and rap1GAP-transfected cells as the more slowly progressing rap1GAP-transfected cells reached G₂ (Figure 4, D and E). Cells were arrested in the G₂ phase as expected with this mitosis inhibitor. These findings are consistent

with a slower progression through the cell cycle of cells transfected with rap1GAP.

Active GTP-Bound Rap1 Is Highly Expressed in SCC

Rap1 activation in normal keratinocytes (HOK), immortalized keratinocytes (IHOK), and human head and neck SCC cells was assayed by the ral-GDS pull-down assay.²¹ Oral and laryngeal SCC cells strongly expressed active rap1, whereas signals of progressively lower intensity were detected in immortalized and normal keratinocytes (Figure 5A). This enhanced activation in SCC is not a function of an increase in total rap1, which was equivalent in normal keratinocytes, immortalized keratinocytes,

Figure 5. Total and active GTP-bound rap1 and rapGAP in SCC cell lines. **A:** Whole-cell lysates isolated from normal keratinocytes (HOK), immortalized keratinocytes (IHOK), and oropharyngeal SCC (UM-SCC-17B) (30 μ g each) were electrophoresed, transferred, and blotted with rap1 (**top** and **middle**) or GAPDH (**bottom**) antibodies. The active form of rap1 in each cell group (300 μ g protein) was retrieved by ral-GDS. GAPDH was used as a loading control. **B:** The percentage of active GTP-bound rap1, normalized to total rap1, was measured by densitometry. **C:** Whole-cell lysates of normal, immortalized keratinocytes and SCC cells (30 μ g each) were immunoblotted with rap1GAP, rap1GAPII, or GAPDH.

and SCC cells [Figure 5, A (middle) and B]. GAPDH was used as a loading control for total rap1. UM-SCC-11A and UM-SCC-14A, two other SCC cell lines also exhibited a high concentration of active rap1 (data not shown). Thus, rap1 is highly activated in SCC cells. Expression of rap1GAP and rap1GAPII was investigated in these cells. Although rap1GAPII was equivalently expressed in normal, immortalized, and malignant keratinocytes, rap1GAP was more strongly expressed in SCC cells and immortalized keratinocytes than in normal keratinocytes (Figure 5C).

Rap1GAP Inhibits Proliferation in SCC Cell Lines

To determine the effects of rap1 on proliferation in SCC, UM-SCC-11A, a representative SCC cell line that is amenable to transfection, was stably transfected with control vector or pcDNA-FLAG-rap1GAP. FLAG-tagged rap1GAP was expressed in the mixed clonal population as shown by immunoblot analysis (Figure 6A, top). The vector control was appropriately negative. GAPDH, used as a loading control, showed equivalent protein loading in both lanes. Consistent with the functional effects of rap1GAP in reducing active GTP-bound rap1, rap1GTP was greater in control cells than in rap1GAP-transfected cells (Figure 6A). Using the trypan blue enumeration assay with the mixed clonal population, we observed a significant decrease in total cell number in cells transfected with rap1GAP compared to the vector control that was apparent as early as 2 days after seeding (Figure 6B, $P < 0.01$). Thus, rap1GAP inhibits proliferation in SCC.

To determine whether inhibition of rap1 activation by rap1GAP involved ERK, whole-cell extracts of rap1GAP- and control vector-transfected SCC cells were prepared and immunoblotted with antibodies to phosphorylated ERK1/2 and total ERK. As shown in Figure 6C (top),

phospho-(active) ERK1/2, normalized to total ERK, was significantly decreased in rap1GAP-transfected cells (Figure 6D, $P < 0.05$). To evaluate whether rap1GAP also inhibits the PI₃K/Akt signaling cascade, whole-cell extracts were immunoblotted with phospho-specific Akt antibodies. As shown in Figure 6, C (third panel) and D, phospho-Akt, normalized to total Akt, was unchanged between rap1GAP- and control vector-transfected cells. Taken together these results suggest that rap1GAP inhibits proliferation via inhibition of ERK activation.

To investigate whether rap1GAP's inhibitory effects on proliferation in UM-SCC-11A were also due to the effects on the cell cycle, cells transfected with pcDNA or rap1GAP were analyzed for cell-cycle distribution in the presence or absence of nocodazole. In the absence of nocodazole treatment, vector control- and rap1GAP-transfected cells exhibited similar cell-cycle profiles (Figure 7A). However, at 6 and 10 hours after nocodazole treatment, UM-SCC-11A stably transfected with rap1GAP had a greater proportion of cells in the S phase and a smaller proportion of cells in the G₂ phase than the vector control (Figure 7A). By 16 hours after nocodazole treatment, the cell-cycle distribution was similar between control vector- and rap1GAP-transfected cells. As shown in Figure 7B, the vector control cells showed a more rapid increase in the G₂ phase than rap1GAP-transfected cells. The disparity is most prominent at 6 and 10 hours after nocodazole treatment. Taken together these findings are consistent with a slower progression through the cell cycle and are similar to those observed with HEK 293T cells (Figure 4).

Lysates from vector control- and rap1GAP (GAP)-transfected cells were prepared a minimum of 72 hours after seeding and analyzed for expression of cell-cycle proteins. The eukaryotic cell cycle is regulated by the action of the cyclin-dependent kinases (cdk) and their

Figure 6. Rap1GAP inhibits proliferation of SCC cells. **A:** Rap1GAP inhibits rap1 activation in SCC cells. Whole-cell lysates (40 μ g each) of a mixed clonal population of pcDNA- or rap1GAP-transfected UM-SCC-11A were electrophoresed, transferred, and exposed to FLAG or rap1 or GAPDH antibodies. The active form of rap1 (rap1^{GTP}) in each cell group (300 μ g each) was retrieved by ral-GDS pull-down and blotted with rap1 antibody. **B:** Rap1GAP retards proliferation of UM-SCC-11A. Mixed clonal populations of UM-SCC-11A cells stably transfected with empty vector (pcDNA) or rap1GAP (GAP) were seeded at 20,000 cells per 60-mm dish, and cell number was measured as described in the Materials and Methods section at days 2, 3, and 4 after seeding. The values represent means of cell number $\pm$ SD based on three wells/data point in a single experiment (* P < 0.01, GAP versus pcDNA). The experiment is representative of three experiments with similar results. **C:** Whole-cell lysates of UM-SCC-11A, transiently transfected with pcDNA and rap1GAP (30 μ g protein each), were electrophoresed and immunoblotted with phospho-ERK1/2, total ERK, phospho-Akt, and Akt antibodies. The data are representative of two independent experiments. **D:** The optical density of phospho-ERK1/2 and phospho-Akt in transfected cells, normalized to total ERK and total Akt, respectively, were expressed as percentage of empty vector control (* P < 0.05 versus pcDNA).

regulatory subunits, the cyclins.²⁸ We observed down-regulation of cyclin D1, cdk6, and cdk4 proteins (Figure 8, A and B) in rap1GAP-transfected cells compared to control vector (pcDNA)-transfected cells. In contrast, cyclin A and cdk2 exhibited no significant change (Figure 8, A and B). The same filter used to detect cdk6 was stripped and reblotted for cdk4 and then GAPDH, as a loading control.

Rap1GAP Inhibits SCC Tumor Growth in Vivo

To determine the effects of rap1 on proliferation of SCC *in vivo*, UM-SCC-11A cells stably expressing rap1GAP or pcDNA vector control (1×10^5 cells each) were injected

subcutaneously into athymic nude mice. The animals were euthanized after 14 days, and tumor weight and volume were quantified. As predicted from the *in vitro* studies, rap1GAP had a significant suppressive effect on tumor growth (Figure 9). Tumor weight and volume (Figure 9, A and B, respectively) were significantly different in rap1GAP-expressing cells (P < 0.01) compared to control vector-transfected cells. The inhibitory effect of rap1GAP on tumor growth is apparent in the significant difference in tumor size between rap1GAP- and control vector-transfected cells, as visualized by the gross appearance of the tumors (Figure 9C). The presence of tumor in the tissue specimens was verified by hematoxylin and eosin (Figure 9, E and F, respectively) and im-

Figure 7. Rap1GAP slows cell-cycle progression of SCC cells. UM-SCC-11A cells stably transfected with pcDNA or pcDNA-FLAG-rap1GAP were cultured in the absence or presence (+N) of nocodazole (200 ng/ml) for 0, 6, 10, and 16 hours. Cell-cycle distribution was determined by flow cytometric analysis of DNA content. **A:** Graphic representation of cell-cycle distribution. The data are representative of two independent experiments with similar results. **B:** Percentage of cells in the G₂ phase of the cell cycle at 0, 6, 10, and 16 hours.

munohistochemical staining of 5- μ m tissue sections (Figure 9, G–J). Tumors expressing rap1GAP and pcDNA control vector exhibited invasive tumor islands in the surrounding connective tissue (Figure 9E). The UM-SCC-11A cells transfected with control vector exhibited closely distributed tumor islands with minimal intervening mesenchymal tissue (Figure 9E, arrows). In contrast, tumors

induced by rap1GAP-expressing cells showed more sparsely distributed tumor islands with larger amounts of intervening mesenchymal tissue (Figure 9F, arrows). Furthermore, keratin pearl formation, a marker of tumor differentiation, was more prominent in control tumors (Figure 9, E and G; small arrows) than in tumors expressing rap1GAP (Figure 9F, small arrows). To verify that tumors induced by rap1GAP-transfected SCC cells continued to express rap1GAP *in vivo*, tissue sections were stained with an anti-FLAG antibody because the rap1GAP cDNA was tagged with a FLAG epitope. Rap1GAP-transfected cells stained positively with the FLAG antibody whereas no staining was observed in the control vector transfected cells (Figure 9, J and I, respectively). As a positive control for antigenicity, cytokeratin, an antigen specific for epithelial cells, was detected in tumors induced by UM-SCC-11A transfected with rap1GAP or control vector (Figure 9, H and G, respectively). Thus, rap1GAP inhibits tumor growth of SCC *in vivo*.

Discussion

In the United States, oral cancer accounts for more deaths annually than cervical cancer, melanoma, or lymphoma.²⁹ Current treatment regimens for SCC are selected according to tumor size and the presence of metastasis. However, even tumors at the earliest stage of disease may vary dramatically in treatment response and recurrence. In this regard, expression of tumor suppressor genes may play an important role in tumor progression. The results presented here suggest that rap1GAP has a tumor suppressor effect in SCC by delaying cell-cycle progression. This is the first report of rap1GAP functioning as a tumor suppressor in human cancer and identifies a new mechanism for growth regulation in SCC. Our findings could have implications for treatment selection and response.

Oropharyngeal SCC cells stably transfected with rap1GAP exhibited decreased proliferation relative to cells containing the control vector. We hypothesized that the inhibition of proliferation is due to a decreased rate of progression through the cell cycle, rather than an arrest of proliferation, because rap1GAP-expressing clones would not have proliferated through >30 population doublings, if rap1GAP mediated cell-cycle arrest. Consistent with this hypothesis, untreated control vector- and rap1GAP-transfected cells exhibit a similar cell-cycle distribution profile whereas on nocodazole treatment, rap1GAP-transfected cells accumulated in the S and G₂ phases more gradually than control cells. These findings are consistent with slower progression through the G₁/S phase of the cell cycle. In support of our findings, rap1, which is inactivated by rap1GAP, is required for cAMP-induced G₁/S transition during mitogenesis in rat thyroid cells.¹⁹ There are other examples of GAP proteins influencing cell-cycle progression. Tuberin/TSC2, a protein with a rap1GAP homology region, inactivates rheb, a rap1-like GTP-binding protein.³⁰ Inactivation of tuberlin decreased the proportion of cells in G₁, consistent with a shortened G₁ phase compared to other phases of the cell

Figure 8. Rap1GAP down-regulates cyclin D1, cdk4, and cdk6. **A:** Whole-cell lysates from control vector (pcDNA)- and rap1GAP (GAP)-transfected UM-SCC-11A cells (30 μ g each) were blotted with antibodies to cyclin D1, cyclin A, cdk6, cdk4, and cdk2. The filter blotted with cdk6 was subsequently stripped and reblotted with GAPDH antibody as a loading control. The data are representative of two independent experiments. **B:** The optical density of cell-cycle proteins, normalized to GAPDH, was expressed as percentage of empty vector control (**P* < 0.01).

Figure 9. Rap1GAP inhibits SCC tumor growth *in vivo*. UM-SCC-11A cells stably transfected with control vector (CV) or FLAG-tagged rapGAP were injected subcutaneously in mice. After 2 weeks, the tumors were harvested, measured in three dimensions (**A**) and weighed (**B**) ($P < 0.01$). **C** and **D**: On gross examination, the tumors were different in size. **E** and **F**: The presence of SCC was confirmed in the tumors by H&E staining. **G** and **H**: The epithelial origin of the tumors was confirmed by immunohistochemical staining for cytokeratin. **J**: Expression of Flag-tagged rap1GAP in rap1GAP-transfected cells was confirmed by immunohistochemical staining with Flag antibody. **I**: The control vector-transfected cells did not stain with Flag antibody. Scale bars: 1 cm (**C**); 100 μ m (**I**).

cycle. In addition to the truncated G₁ phase of the cell cycle, inhibition of tuberlin/TSC2 up-regulated cyclin D1 expression. Data in the present study also suggest that rap1GAP delays cell-cycle progression, possibly via inhibition of ERK activation. Consistent with these findings, ERK has been shown to induce cyclin D1.³¹ Because rap1GAP shifts rap1 from the active GTP-bound form to inactive GDP-bound rap1, these observations suggest that an intact rap1 GTP-GDP switch is required for cell-cycle progression and proliferation in oropharyngeal SCC and HEK 293T cells, a transformed cell line.

Based on the observations that rap1GAP enhanced proliferation in nonmalignant keratinocytes,¹⁷ we postulated that rap1GAP could also have effects on proliferation in malignant keratinocytes. In contrast to normal keratinocytes, inhibition of rap1 in SCC leads to decreased proliferation in malignant keratinocytes. Consistent with these findings, previous studies have shown that the same signaling pathway may be anti-mitogenic in normal keratinocytes but proproliferative in SCC. For example, ERK up-regulates proliferation in SCC but promotes differentiation in normal keratinocytes.^{32,33} In SCC, an increase in the ratio of ERK to p38 MAPK facilitates proliferation whereas in normal keratinocytes, an increase in ERK with a concomitant decrease in the 95-kd isoform of B-raf promotes differentiation.^{32,33} In the present study, rap1GAP inhibited ERK activation in SCC, suggesting that the anti-proliferative effect occurs via the ERK signaling pathway.

The partial inhibition of ERK activation (Figure 6C) suggests that other signaling intermediates, such as ras, regulate ERK activation in SCC. Although ras is one of the most common oncogenic proteins in humans, it is rarely mutated in SCC in the Western Hemisphere.³⁴ SCCs exhibit enhanced secretion of several cytokines, such as EGF, which induce ERK activation via a ras-dependent signaling mechanism.³⁵ Hence, disparities in SCC behavior may be attributable to the extent to which cytokines are overexpressed and signaling intermediates that regulate proliferation are disrupted. Some of these overexpressed cytokines also stimulate rap1 (unpublished observations), which may explain why active GTP-bound rap1 is prominent in SCC cells despite rap1GAP expression. Alternatively, it is possible that rap1GAP in SCC is inactive, although inactivating mutations of this protein have not been reported. The rap1GAP peptides of higher molecular mass (Figure 5) may represent isoforms that have been inactivated by phosphorylation, leading to an increase in active GTP-bound rap1.

Tuberlin suppresses tumorigenicity of TSC2-deficient Eker rat cells.^{36,37} Deficiency of SPA-1, a rap1GAP homologue, is associated with the development of leukemias and other disorders of the hematopoietic system in mice.⁸ In SCC, overexpression of rap1GAP inhibits proliferation *in vitro* and tumor growth *in vivo* and appears to inhibit keratin pearl formation as well. Studies presented here indicate that aberrant signaling pathways that are engaged in SCC are repressed by rap1GAP, which acts as a tumor suppressor protein. The proposed model of rap1GAP-mediated growth suppression is diagrammatically represented in Figure 10.

Figure 10. Proposed model for interaction between rap1GAP and downstream effectors in the regulation of proliferation in squamous cell carcinoma.

In summary, findings of this study indicate that inactivation of rap1 inhibits proliferation in human oropharyngeal cancer cell lines and tumor growth *in vivo*. These inhibitory effects on proliferation are due to a delay in the G₁/S transition of the cell cycle. These novel findings show that rap1GAP acts as a tumor suppressor in SCC. If rap1GAP is a tumor suppressor protein, then its expression in SCCs would be predictive of slower growing lesions and possibly a more favorable prognosis. Studies relating rap1GAP expression to clinical outcome and treatment response of SCC could determine whether this protein is a biomarker for prognostically favorable SCCs.

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