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13. ABSTRACT ***(Maximum 200 Words)***

This project focuses on the biology of the Rak protein tyrosine kinase in human breast cancer. Rak is a 54 kDa protein tyrosine kinase expressed in epithelial cells. Rak resembles the proto-oncogene Src structurally, but Rak lacks an amino-terminal myristylation site and localizes to the nuclear and perinuclear regions of the cell. We report here that expression of Rak in breast cancer cells inhibits growth and causes G_1 arrest of the cell cycle. This growth inhibition is kinase-dependent, but does not require the Rak SH2 or SH3 domains. Rak also binds to the pRb retinoblastoma tumor suppressor protein, but Rak inhibits growth even in cells that lack pRb. These results are consistent with Rak functioning as a regulator of cell growth that is distinct from the Src-related kinase family. Our other studies are examining the expression of Rak in human breast cancer specimens using monoclonal antibodies as well as Taqman analysis of mRNA expression. We are also producing an adenoviral Rak construct to allow robust expression of Rak in breast cancer cells to further assess the growth inhibitory effect and to characterize the biology of this growth inhibitory protein tyrosine kinase.

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FOREWORD

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ABSTRACT

This project focuses on the biology of the Rak protein tyrosine kinase in human breast cancer. Rak is a 54 kDa protein tyrosine kinase expressed in epithelial cells. Rak resembles the proto-oncogene Src structurally, but Rak lacks an amino-terminal myristylation site and localizes to the nuclear and perinuclear regions of the cell. We report here that expression of Rak in breast cancer cells inhibits growth and causes G_1 arrest of the cell cycle. This growth inhibition is kinase-dependent, but does not require the Rak SH2 or SH3 domains. Rak also binds to the pRb Retinoblastoma tumor suppressor protein, but Rak inhibits growth even in cells that lack pRb. These results are consistent with Rak functioning as a regulator of cell growth that is distinct from the Src-related kinase family. Our other studies are examining the expression of Rak in human breast cancer specimens using monoclonal antibodies as well as Taqman analysis of mRNA expression. We are also producing an adenoviral Rak construct to allow robust expression of Rak in breast cancer cells to further assess the growth inhibitory effect and to characterize the biology of this growth inhibitory protein tyrosine kinase.

PROGRESS REPORT

This research project focuses on protein tyrosine kinases in human breast cancer. Specifically, we are studying the role of the novel tyrosine kinase, Rak, which our laboratory first identified several years ago (Cance et al., 1994).

Rak is a 54kd cytoplasmic tyrosine kinase that is a member of the *src* family. It encodes both SH2 and SH3 domains, a kinase domain, and a bipartate nuclear localization signal. Unlike other members of the *src* family, Rak lacks the myristylation signal that localizes the protein to the inner leaf of the cellular membrane. Cell fractionation studies show Rak to pellet with the nucleus. This research program has focused on the role of the Rak tyrosine kinase in human breast cancer, and we have focused on characterizing the biochemical effects of Rak expression in breast cancer cells as well as the resultant phenotypic effects to determine if Rak is a therapeutic target in human breast cancer.

TECHNICAL OBJECTIVE 1

1. Characterization of the expression of Rak in human breast cancer cells.

A. Model Systems

In this section, we will review the model systems that we have used to express Rak in human breast cancer cells. Please see attached manuscript which is a final draft before submitting to *Oncogene* in August 2000.

1. Green fluorescence protein (GFP)-Rak (GFP-Rak)

We have constructed an expression vector which contains the green fluorescence protein fused to the Rak DNA. We have designed our constructs such that Rak is translated prior to the green florescence protein. The construct was then used for transfection into cell lines. Expression has been confirmed in multiple cell lines including COS7, BT474, MCF10, 293, SKBR3, MCF7, U2OS, and SAOS. The majority of the expression studies have been carried out in BT474 cells although the expression pattern is similar in all cells tested. Please see attached manuscript for details (Appendix I) .

2. Adenoviral-Rak

We have been attempting to create an adenoviral construct containing HA-tagged Rak. We had been unsuccessful with our original attempts which used Flag-tagged Rak and we have recently cloned Rak into the pShuttle vector for the pAdEasy system which has been reported to be a more straightforward method of obtaining adenoviral constructs. We have obtained recombinant plasmids in this system but were unable to detect Rak expression in the lysates. We then expressed Rak into 293 cells and COS-7 cells and were able to demonstrate a robust gene expression. (Appendix II). In these experiments, we see expression of the pShuttle-Rak in 293 cells, detected by anti-HA epitope tag (Panel A) and anti-Rak 1.61 (Panel B). The UNC Viral Core Facility is currently amplifying the virus again in an attempt to develop recombinant virus.

Our concern in this system is that the growth inhibition of Rak may in fact inhibit the adequate production of virus. These issues are currently being analyzed the Gene Therapy Core Facility. However, the pShuttle vector for this adenoviral system did give excellent gene expression in the 293 cells, and we are currently testing the ability of the vector itself to transiently express in BT474 cells. We need to achieve a higher percentage of infection and are thus moving away from the transient transfection approaches in our manuscript to an approach which would express Rak in over 90% of the cells.

3. Mutagenesis of the kinase domain and amino terminal deletion construct analysis

We have successfully mutated these areas of Rak. Please see attached manuscripts for details (Appendix I).

4. Phage display - Baculovirus and Intein systems

We are pursuing the phage display approach in order to define binding partners of Rak. In order to use this technique, we must produce recombinant protein. We have cloned Rak into New England BioLab's Intein vector but were unable to achieve significant protein expression in this system. We have cloned Rak into a baculovirus vector and are currently obtaining recombinant virus that we can then use to produce Rak protein in the Sf9 cells. We have an agreement with Novalon Pharmaceuticals to perform the phage display once we have obtained adequate Rak expression.

2. Rak is Growth Inhibitory in Human Breast Cancer Cells.

1. Please see attached manuscript for details of Rak's growth inhibitory properties (Appendix I).

2. Rak Transfected Cells Develop Filopodia

As noted in our previous report, Rak transfected cells began to develop filopodia type cytoplasmic extensions. This has been seen in approximately 20% of the transfected cells. We initially hypothesized that this was related to an interaction of Rak with the Rac pathway. This was based on our initial findings where cotransfection of Rak with dominant-negative Rac, caused no filopodia in the breast cancer cells. In collaboration with Dr. Keith Burridge at UNC Lineberger Comprehensive Cancer Center, we have tested this hypothesis and were not able to demonstrate a direct interaction between these two molecules. We do not have a good explanation for the development of the filopodia, particularly if Rak is growth inhibitory, but we are not pursuing this further investigation as part of the Rak project.

TECHNICAL OBJECTIVE 2

1. Development and Characterization of Monoclonal Antibodies to the Rak Protein

We have developed monoclonal antibody 1.61 which recognizes Rak on Western blot analyses. We have tested Rak on Western blots of human tumor samples, but found that they do not express significant amounts of Rak to be detected by this antibody. This antibody works very well in tissue culture systems, particularly when we have transiently overexpressed Rak. For example, we have used the adenoviral shuttle vector expressed in 293 cells to generate lysate to test the antibody.

We are collaborating with Dr. Funda Meric at MD Anderson Cancer Center on Rak and have supplied them with this antibody for their usage.

2. Development of Taqman Studies of Rak Expression in Human Breast Cancer

Because our monoclonal antibodies did not work well in human breast tumors, we wished to ask whether the mRNA expression was changed between normal breast epithelium and breast cancer. In collaboration with Dr. Benjamin Calvo, we have developed Taqman PCR-based quantitative mRNA protocols. We have synthesized a Rak specific primer for the use in this assay and are currently testing pilot experiments of Taqman in human breast cancer specimens.

KEY RESEARCH ACCOMPLISHMENTS

- Rak is growth inhibitory in human breast cancer cells.
- Rak causes a G1 arrest of the cell cycle.
- Rak's growth inhibition is kinase dependent but does not Rak SH2 or SH3 domains.
- PShuttle-Rak is expressed at high levels in 293 cells and will be the basis for the development of adenoviral Rak contructs.
- Rak appears to be expressed at low levels in approximately 30% of human breast cancers.

REPORTABLE OUTCOMES

1. DOD Era of Hope Abstract
2. Attached manuscript – to be submitted to *Oncogene* in August 2000 (Appendix I)

CONCLUSIONS

Rak is a growth inhibitory gene in human breast cancer causing a G1 arrest on the cell cycle. The growth inhibition of Rak is kinase dependent but does not require the Rak SH2 or SH3 domains. Rak binds to the pRb retinoblastoma tumor suppressor protein but Rak inhibits growth in the cells that lack pRb. These results are consistent with Rak functioning as a regulator of cell growth that is distinct from the Src related kinase family.

REFERENCES

Cance, W.G., Craven, R.J., Bergman, M., Xu, L., Alitalo, K. & Liu, E.T. (1994). *Cell Growth Differ*, **5,** 1347-55.

Also please see attached manuscript—Appendix I.

APPENDICES

I. The Rak tyrosine kinase inhibits growth of human breast cancer cells. Draft of paper.

II. pShuttle expression of Rak into 293 cells and COS-7 cells.

DRAFT

The Rak tyrosine kinase inhibits growth of human breast cancer cells

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ABSTRACT

Rak is a 54 kDa protein tyrosine kinase expressed in epithelial cells. Rak resembles the proto-oncogene Src structurally, but Rak lacks an amino-terminal myristylation site and localizes to the nuclear and perinuclear regions of the cell. We report here that expression of Rak in breast cancer cells inhibits growth and causes G_1 arrest of the cell cycle. This growth inhibition is kinase-dependent, but does not require the Rak SH2 or SH3 domains. Rak also binds to the pRb Retinoblastoma tumor suppressor protein, but Rak inhibits growth even in cells that lack pRb. These results are consistent with Rak functioning as a regulator of cell growth that is distinct from the Src-related kinase family.

MAIN TEXT

Protein tyrosine kinases play key roles in signal transduction and influence a number of cellular processes that maintain the balance between proliferation, differentiation, senescence, and apoptosis. We previously conducted a PCR-based screen for novel protein kinases in breast cancer cell lines and identified Rak (simultaneously identified as FRK (Lee et al., 1994)). Rak is a member of a sub-family of Src-related tyrosine kinases that includes Sik (Src-related intestinal kinase)(Vasioukhin et al., 1995), Brk (breast tumor kinase)(Mitchell et al., 1994), Gtk (Gastrointestinal tyrosine kinase, rat) (Sunitha et al., 1999), and Bsk/Iyk (intestinal tyrosine kinase, mouse) (Thuveson et al., 1995). The distinguishing features of this sub-family of kinases is that they are all expressed primarily in epithelial tissues and share greater identity within the group than to Src itself. The Rak-related kinases are distinct from the CSK (carboxy-terminal src kinase) sub-family because they contain a tyrosine near their carboxy-termini.

The Rak-related kinases all contain Src homology 2 and 3 domains (SH2 and SH3) at their amino-termini. SH2 domains bind to phosphorylated tyrosine residues, while SH3 domains associate with proline-rich sequences of target proteins. These domains are involved in both inter-molecular associations that regulate signaling cascades and intra-molecular associations that auto-regulate protein kinase activity (Cance et al., 1994). The role of these domains in Rak are unknown. At its carboxy-

terminus, Rak has a kinase domain and associated autophosphorylation activity. In addition, the carboxy-terminal tyrosine of Rak is phosphorylated *in vitro* by CSK, suggesting that this may be a regulatory site (Cance et al., 1994). While Rak resembles Src structurally, Rak lacks the myristylation signal that localizes Src to the cell membrane. Instead, Rak contains a putative bipartite nuclear localization signal within its SH2 domain and co-fractionates with nuclear proteins in some cell lines (Cance et al., 1994). Additionally, Rak associates with pRb during the G_1 and S phases of the cell cycle by interacting with the A/B pocket of pRb, and endogenous Rak is elevated during the G_1 phase of the cell cycle (Craven et al., 1995).

To investigate the biologic effects of Rak expression in breast cancer cells, we created a series of Rak expression constructs fused in frame with the Flag epitope at the amino terminus and the green fluorescent protein (GFP) at the carboxy terminus. Rak was transfected into BT474 cells, which were originally derived from a solid invasive ductal carcinoma of the breast. Rak-GFP was efficiently expressed in these cells as an 80 kDa protein (Figure 1B, lane 3) with autophosphorylation activity (Figure 1C, lane 2) that localized to the perinuclear region of the cell (Figure 1D, second row, right panel). We have previously shown that Rak inhibits the growth of NIH3T3 cells (Craven et al., 1995), and we found that Rak-GFP expression is excluded during extended growth of BT474 cells (data not shown) and that Rak inhibits the colony formation of these cells (Table I).

This lab has previously reported that Rak localizes to the nucleus in COS-7 cells, whereas Rak-GFP localized to the perinuclear region in the BT474 cell line. To determine whether this perinuclear expression pattern is a general property of breast cancer cell lines, we transfected several different cell lines with Rak-GFP, including SK-BR-3, BT20, MCF7, U2OS, and SAOS-2, and all demonstrated a peri-nuclear localization (data not shown). It is unlikely that the GFP fusion partner of Rak altered the localization of Rak-GFP, because a separate construct containing Rak fused in frame with the nine residue Flag epitope demonstrated a perinuclear distribution by immunofluorescence (data not shown). In our previous analysis, endogenous Rak localized to the nucleus in COS-7 cells (Cance et al., 1994), but we have identified endogenous Rak in the perinuclear region in other cell lines. A similar pattern of staining

in the nucleus and cytoplasm was also reported for the murine Rak homologue Iyk (Berclaz et al., 2000). We cannot exclude the possibility that the overexpression system used here somehow selects for the latter localization pattern.

To determine which domains of Rak contribute to growth inhibition, we constructed a series of mutations within the Rak coding sequence, fused them to GFP, and determined their localization and effect on growth. Rak mutants lacking an SH2 domain (Rak-SH2Δ), an SH3 domain (Rak-SH3Δ), or a kinase-inactive mutant (Rak-KD) were expressed in BT474 cells and analyzed by western blot and kinase assay. Each of the mutants was efficiently expressed (Figure 1B) and the Rak-SH2Δ and Rak-SH3Δ mutants possessed auto-phosphorylation activity *in vitro* (Figure 1C). In contrast, the Rak-KD mutant lacked autophosphorylation activity (Figure 1C). Each of the mutants localized to the perinuclear region, similar to wild-type Rak (Figure 1D), and the Rak-SH2Δ and Rak-SH3Δ mutants inhibited colony formation in a similar manner to wild-type Rak (Table I). However, the Rak-KD kinase-inactive mutant did not inhibit colony formation in BT474 cells, indicating that kinase activity is required for growth suppressing activity (Table I).

The colony formation assay indicated that Rak had a growth inhibitory activity, but revealed little about the nature of this activity. For this reason, we performed transient transfections and measured the incorporation of BrdU into transfected and non-transfected cells. Rak transfectants were visible as green cells, while BrdU incorporation was detected with a red rhodamine-conjugated secondary antibody. BrdU was efficiently incorporated into cells transfected with a control plasmid (Figure 2A and 2B, upper left panel). In contrast, Rak-GFP expression blocked BrdU incorporation (detected as green cells which do not stain red for BrdU incorporation, Figure 2A and 2B, right upper panel), while untransfected cells continued to incorporate BrdU normally (detected as red nuclei in non-fluorescent cells). This disruption of BrdU incorporation was also detected in Rak-SH2Δ and Rak-SH3Δ mutants (Figure 2A and 2B, second row, right panel and bottom panel, respectively), while cells transfected with the Rak-KD mutant incorporated BrdU at levels approaching the vector control ((Figure 2A and 2B, second row, left panel).

Inhibition of BrdU incorporation suggested that Rak might arrest BT474 cells in the G_1 phase of the cell cycle. However, G_1 arrest was difficult to determine by FACS analysis because the majority of BT474 cells are in G_1 normally. To circumvent this problem, we transfected cells, then treated them with colcemid, a microtubule-disrupting agent that arrests cells in G_2/M, and performed FACS analysis. As expected, cells transfected with a control vector and treated with colcemid accumulated in G_2/M, with only 11% of cells maintained in G_1 (Figure 3A and B). In contrast, 47% of Rak-transfected cells remained in G_1 (Figure 3A and C), as did the SH2Δ and SH3Δ mutants (Figure 3E and F). As expected, the Rak-KD mutant resembled the control vector, with only 16% of cells arrested in G_1 and the majority in G_2/M (Figure 3D). This result was not specific to colcemid treatment. Cells treated with 1nM Taxol (a microtubule stabilizing agent that also causes G_2/M arrest) and transfected with Rak had 32% (±10) of transfected cells in the G_1 phase of the cell cycle, while 8% (±2) of control transfected cells remained in G_1 after Taxol treatment. Thus, by two separate criteria, we conclude that Rak arrests BT474 breast cancer cells before DNA synthesis in the G_1 phase of the cell cycle.

We have previously reported that Rak binds to the tumor suppressor pRb *in vitro* and *in vivo* (Craven et al., 1995), and we wished to determine whether this interaction contributed to the growth suppression by Rak in BT474 cells. Because there are no clearly defined breast cancer cell lines that are wild-type or mutated for pRb, we resorted to Rb^+ U2OS and Rb^- SAOS2 osteosarcoma cells. Transfection with the p16 gene served as a positive control, because p16 will arrest the pRb positive U2OS cells in G_1, but will have no effect on the pRb negative SAOS2 cells [REF]. In a similar experiment to the one described above, cells were transfected with a vector control, Rak-GFP, or p16, and then treated with colcemid and analyzed by flow cytometry. As expected, U2OS cells transfected with p16 arrested in the G_1 phase, but pRb^- SAOS2 cells did not (Figure 4a, right panels and solid line). In contrast, Rak-GFP-transfection arrested SAOS-2 cells in G_1 lines (Figure 4, middle panels and solid line), indicating that pRb is not required for Rak-mediated growth arrest. Instead, the pRb^+ cell line U2OS arrested poorly in G_1 in response to Rak expression (Figure 4a, center top panel), suggesting that pRb might even counteract the effects of Rak-mediated growth arrest. The ability of Rak-GFP to arrest

growth in the absence of pRb is consistent with earlier results demonstrating that deletion of the Rak SH3 domain, which interacts with pRb, did not affect the ability to arrest growth (Table 1 and Figure 2F).

In this report, we have further refined the relationship between Rak and the other Src-related kinases. While Src increases the growth and adhesive properties of BT474 cells (Yang, Xu, Park, and Cance, unpublished observations), Rak inhibited their growth. This growth inhibition required the kinase domain of Rak, suggesting that Rak might inhibit growth by phosphorylating a target protein in the nuclear membrane or Golgi. Given the growth inhibitory properties of Rak, it is notable that Rak expression was lost in 3/9 human breast tumors (Cance et al., 1993) and Iyk expression was undetectable in a panel of human breast tumors (Berclaz et al., 2000). Taken together, these findings suggest that an improved understanding of the Rak signaling cascade might lead to alternate approaches to limit the proliferation of breast cancer cells.

FIGURE LEGENDS

FIGURE 1. Expression and localization of Rak and various deletion mutants in BT474 cells. A. Cartoon showing the structure of Rak and the three mutants used in this study. F, Flag epitope tag; SH3, Src homology domain 3; SH2, Src homology domain 2; GFP, Green fluorescent protein. B. Western blot showing expression of Rak-GFP and various mutations in BT474 cells, detected with an antibody to GFP (Clontech). Lane 1, untransfected cells; lane 2, vector control; lane 3, Rak-GFP; lane 4, Rak-KD-GFP (a kinase-inactive mutant); lane 5, Rak-SH2Δ-GFP; and lane 6, Rak-SH3Δ-GFP. C. Immunoprecipitation and kinase assay of Rak-GFP. Transfected cells were lysed and Rak-GFP was immunoprecipitated with the M2 monoclonal antibody to the Flag epitope tag (Sigma). Immunoprecipitates were either analyzed by Western blot (lower panel), or *in vitro* kinase assay (upper panel). The Rak-GFP, Rak-SH2Δ-GFP, and Rak-SH3Δ-GFP proteins possessed autophosphorylation activity, while the Rak-KD-GFP protein did not. The order of loading was the same as in panel B, above. D. Perinuclear localization of Rak and various deletion mutants. Cells were transfected and analyzed by either bright-field microscopy (left panels) or immunofluorescence (right panels).

METHODS: The Rak coding sequence was amplified by PCR from a plasmid containing the full Rak cDNA with a 5' flag epitope (met-asp-tyr-lys-asp-asp-asp-asp-lys) with KpnI and SalI sites incorporated into the oligonucleotide primers. Rak was inserted into the same sites of the GFP-N1 vector (Clontech), and the sequence was confirmed by automated sequencing. Deletion of the SH2 and SH3 domains and the K262R point mutation were created from the Rak-GFP construct using the ExSite mutagenesis kit (Stratagene). The SH2 domain deletion (SH2Δ) was created by deletion of amino acids 116-198, the SH3 domain deletion (SH3Δ) created by deletion of amino acids 47-108. Transfection, lysis, and western blot or immunoprecipitation of BT474 cells has been previously described (Xu '99). Cells were detected by immunofluorescence using a Zeiss fluorescent microscope.

FIGURE 2. Rak expression inhibits BrdU incorporation. Rak-GFP, or various mutants of Rak, were transfected into BT474 cells and exposed to BrdU. Cells were then stained for BrdU uptake (red) or GFP (green) in Panel A, and the percentage of cells incorporating BrdU divided by the total number of transfected cells was determined (B). The identity of the transfected constructs are shown.

METHODS: At 24 hours post-transfection, 30μM BrdU was added to the media and incubated for an additional 3 hours. Cells were fixed in 70% ice cold ethanol for 30 minutes, washed with 1X PBS, and then fixed in 1% paraformaldehyde 0.05% Tween-20 overnight at 4°. After treatment with 100 units/ml of DNase (RQ1 Gibco) for 1 hour at room temperature, cells were immunostained with α-BrdU antibody (Oncogene Science) at 4° overnight and rhodamine conjugated goat anti-mouse secondary antibody for 1 hour at room temperature. Cells were visualized using a fluorescent microscope with a dual filter and percentage of transfected cells incorporating BrdU determined by manual counting.

As vector control, we used a membrane anchored GFP construct (GFP-βIΣII spectrin) created specifically to allow flow cytometric DNA content analysis of GFP labeled cells. The cytoplasmically located GFP molecule alone is only 27 kDa (239 amino acids) and leaches from the cell due to the membrane permiabilization that occurs with the ethanol fixation required for quantitative DNA analysis. The GFP-βIΣII spectrin

control vector (designated as VC in the text) was a kind gift from Dr. Timothy Collins of the Lineberger Comprehensive Cancer Center (Chapel Hill, NC).

FIGURE 3. Rak expression causes an accumulation of cells in the G_1 phase of the cell cycle. Transfected cells were treated with colcemid and analyzed by flow cytometry. Cells transfected with a GFP-Spectrin plasmid accumulated in the G2/M phase of the cell cycle (Panel B), while cells transfected with Rak-GFP, Rak-SH2Δ-GFP, or Rak-SH3Δ-GFP remained in G_1 (Panels C, E, and F). A kinase-deficient Rak mutant Rak-KD-GFP did not cause G_1 arrest, and transfected cells accumulated in G_2/M. The results are summarized in Panel A.

METHODS: Cells were transfected and incubated with complete media for 24 hours, then treated with 50 ng/ml colcemid for an additional 18 hours. One million cells were trypsinized, washed with cold PBS, and fixed in 70% ice-cold ethanol for at least 2 hours at –20°. Cells were again washed with cold PBS and resuspended in 0.5 ml of PBS containing 10μg/ml RNase (Qiagen), 1% BSA, 0.1% Tween-20, and 100 μg/ml of PI (Boehringer Manheim). Cells (>30,000 cells/sample) were analyzed on a FACScan flow cytometer (Becton-Dickinson) and cell cycle distribution determined with ModFit software.

FIGURE 4. Rak does not require pRb to initiate G_1 arrest. Rb^+ U2OS cells (top panel) and Rb^- Saos-2 cells were transfected and treated with colcemid, similar to Figure 3. Both cell lines transfected with a control vector arrested in G_2/M (left panels), while Rak-GFP-transfected cells remained in G_1 (center panels). Cells transfected with the cell cycle inhibitor p16 arrested only in the presence of Rb (right panels). The histogram of the vector control cells is plotted as a dashed line for reference, while the histograms of Rak-GFP and p16-expressing cells are plotted as solid lines.

METHODS: Transfections and cell cycle analysis were performed exactly as described in Figure 3. U2OS and Saos-2 cells, and the p16 expression plasmid were kind gifts of Dr. Wendell Yarborough (University of North Carolina).

Table I.

% fluorescent cells

	Day 1	**Day 2**	**Day 3**
pGFP-N1	35	35	27
pRak-GFP	24	6	2

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FIGURE 1

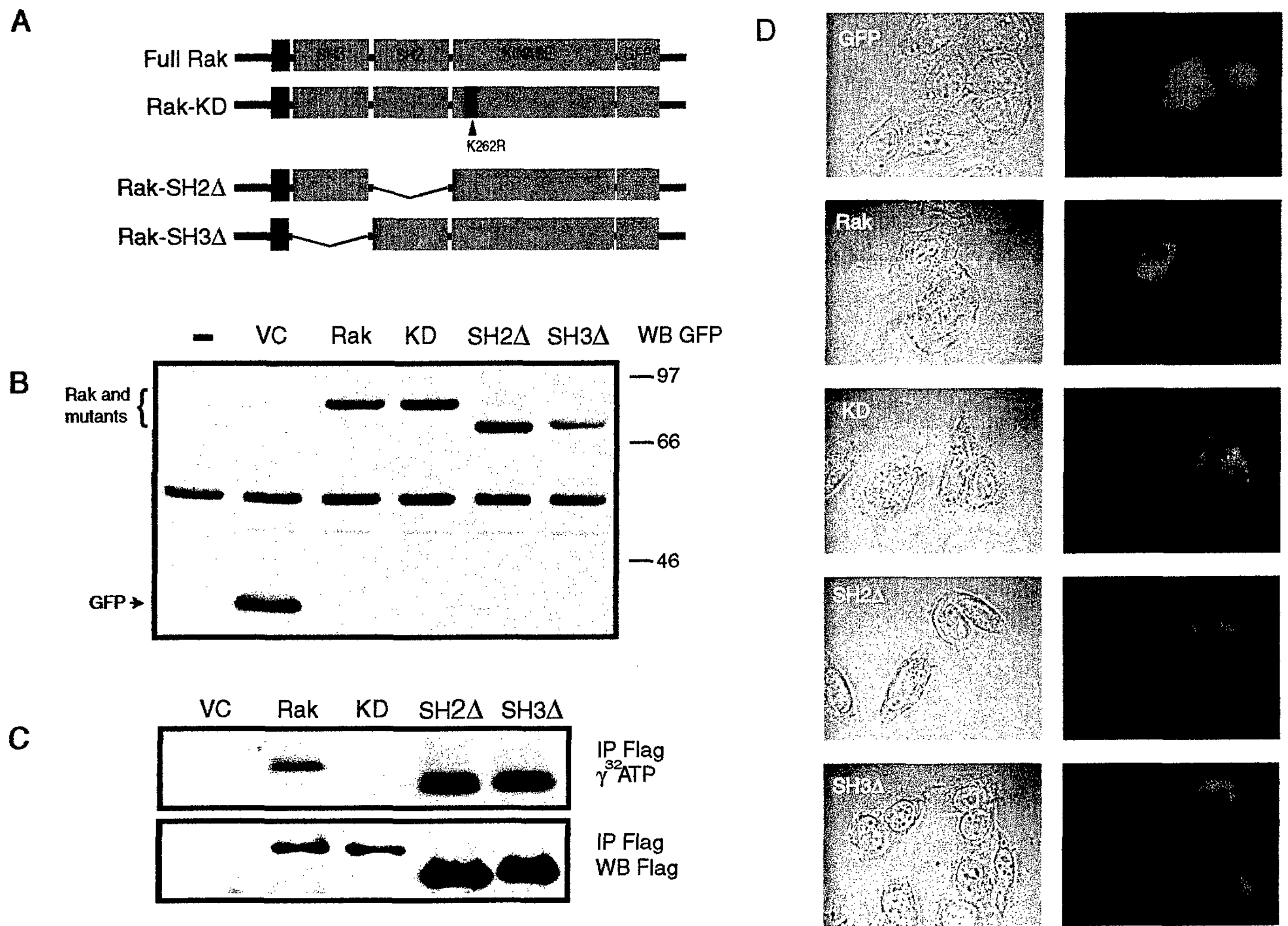
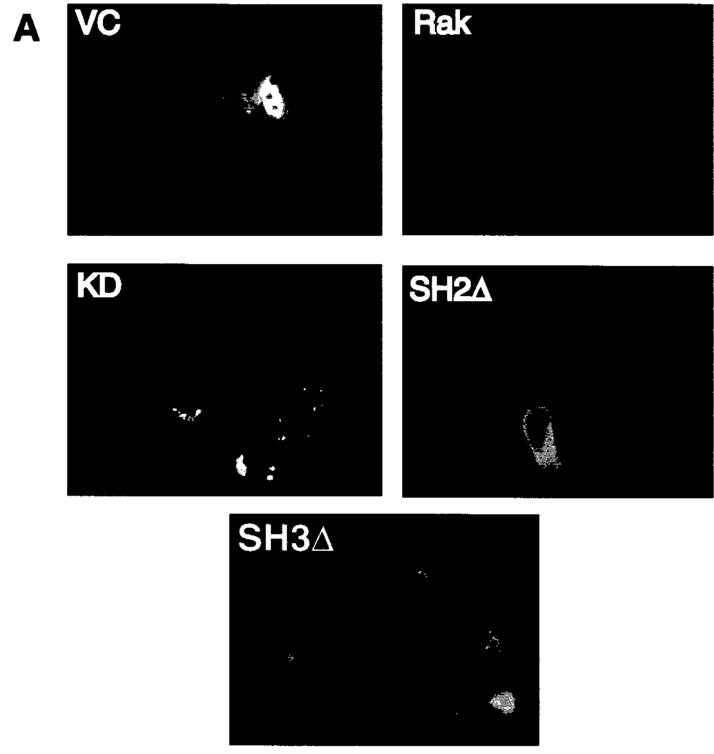


FIGURE 2



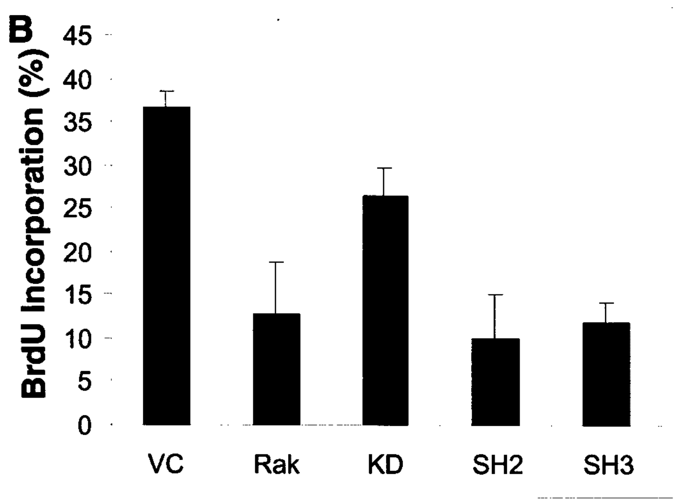
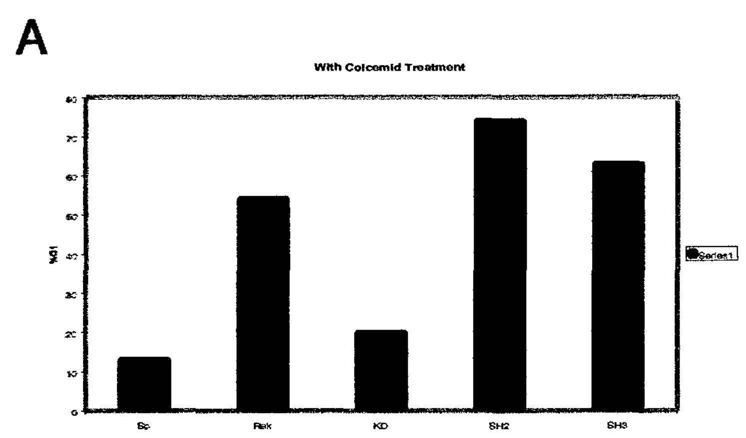


FIGURE 3

A



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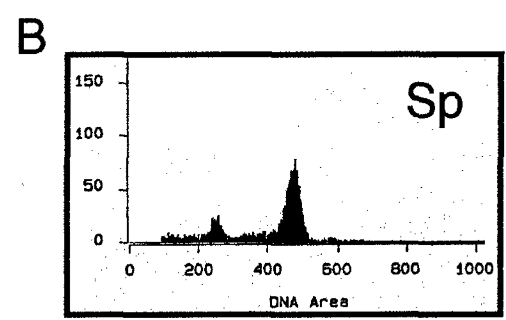

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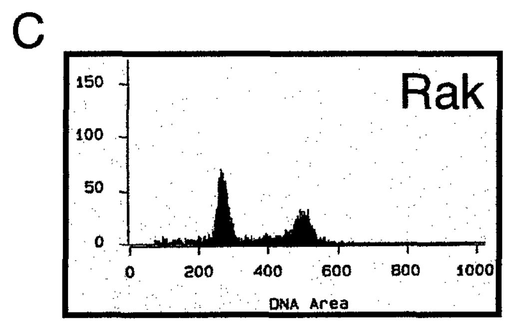

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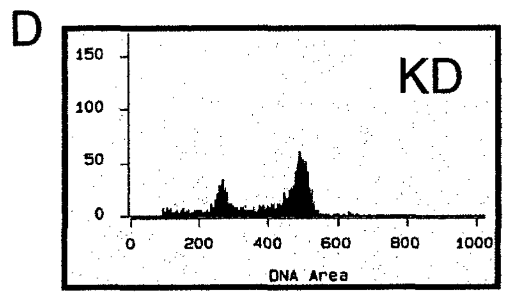

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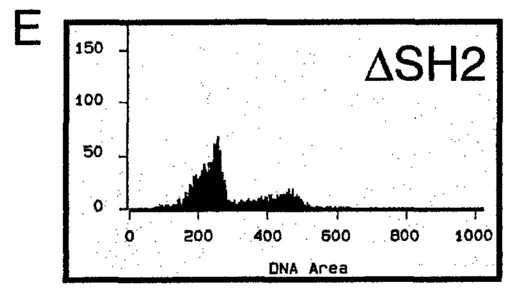

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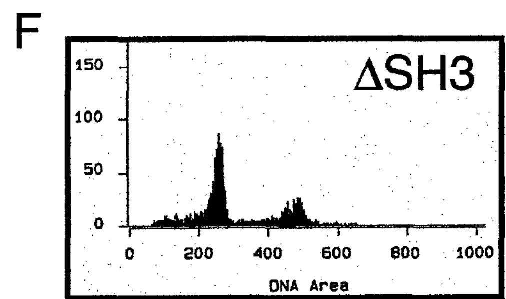

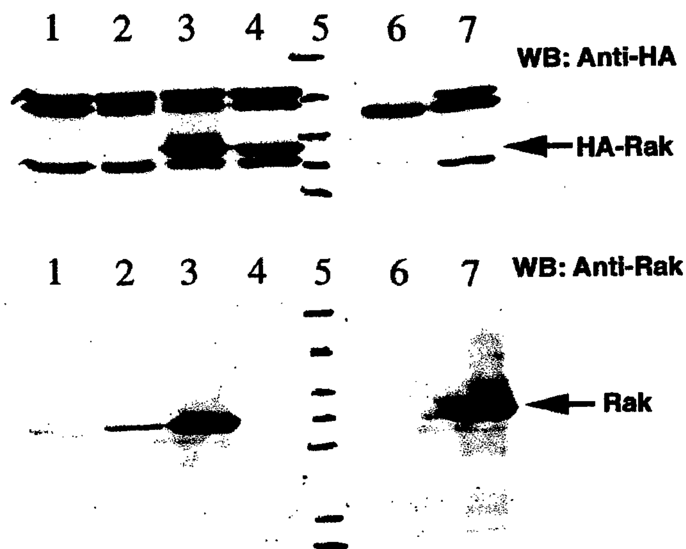

Panel A Western blot Probe with HA Antibody
Lane 1. 293 cells 24 hours
Lane 2. 293 cells 36 hours
Lane 3. 293 Rak 24 hours
Lane 4. 293 Rak 36 hours
Lane 5. Marker
Lane 6. Cos 7 Rak
Lane 7. BT474 Rak

Panel B Western blot Probe with Rak antibody
Lane 1. BT474 Rak
Lane 2. Cos 7 Rak
Lane 3. 293 Rak 36 hours
Lane 4. 293 cells 24 hours
Lane 5. Marker
Lane 6. 293 cells 36 hours
Lane 7. 293 Rak 24 hours

DEPARTMENT OF THE ARMY
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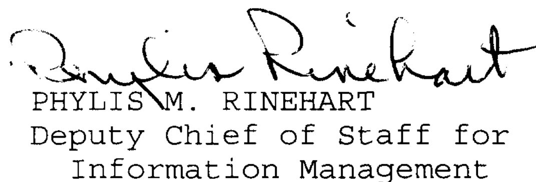

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