Using loss-of-function mutants of Ros and inducible epidermal growth factor receptor-Ros chimeras we investigated the role of various signaling pathways in Ros-induced cell transformation. Inhibition of the mitogen-activated protein kinase (MAPK) pathway with the MEK (MAP/extracellular signal-regulated kinase) inhibitor PD98059 had little effect on the Ros-induced monolayer and anchorage-independent growth of chicken embryo fibroblasts and NIH3T3 cells even though more than 70% of the MAPK was inhibited. In contrast, inhibiting the phosphatidylinositol 3-kinase (PI3K) pathway with the drug LY294002, a dominant negative mutant of PI3K, p85, or the phosphatidylinositol phosphatase PTEN (phosphatase and tensin homologue deleted in chromosome ten) resulted in a dramatic reduction of v-Ros- and epidermal growth factor receptor-Ros-promoted anchorage-independent growth of chicken embryo fibroblasts and NIH3T3 cells, respectively. Parallel and downstream components of PI3K signaling such as the Rho family GTPases (Rac, Rho, Cdc42) and the survival factor Akt were all shown to contribute to ROS-induced anchorage-independent growth, although Rac appeared to be less important for Ros-induced colony formation in NIH3T3 cells. Furthermore, the transformation-attenuated v-Ros mutants F419 and DI could be complemented by constitutively active mutants of PI3K and Akt. Finally, we found that overexpressing a constitutively active mutant of STAT3 (STAT3C) conferred a resistance to the inhibition of ROS-induced anchorage-independent growth by LY294002, suggesting a possible overlap of functions between PI3K and STAT3 signaling in mediating ROS-induced anchorage-independent growth.

The proto-oncogene c-Ros encodes a receptor protein-tyrosine kinase (RPT kinase) that shares structural homology with the sevenless protein of Drosophila melanogaster. In addition, the protein-tyrosine kinase (PT kinase) domain of c-Ros shares greater sequence and structural homology with those of the insulin receptor family members than any other RPT kinases. Although its ligand remains unknown, c-Ros expression is specific and highly regulated. c-Ros has been found to be expressed in a variety of organs/tissues, with relatively high levels in the kidney and intestine. c-Ros protein has been shown to be localized in the epithelial cells of the kidney collecting duct and intestinal villi in chicken. v-Ros is the oncogenic form of c-Ros and was identified as the transforming gene of the avian sarcoma virus UR2. Comparison of the v-Ros oncogene with c-Ros revealed that all but 21 nucleotides immediately upstream of the transmembrane domain of c-Ros were deleted and replaced by the 150 amino acids of the retroviral p19 gag sequence. Two UR2 mutants, F419 and DI, were shown to retain the PT kinase activity and growth-stimulating ability of cells maintained in monolayer but not in anchorage-independent conditions. F419 has a tyrosine to phenylalanine mutation at amino acid 419, whereas DI has a deletion of the unique 6-amino acid insertion located 3 amino acids downstream of the predicted catalytic loop of the PT kinase domain. Previous analysis revealed that F419 is impaired in its ability to activate insulin receptor substrate-1 and PI3 kinase, which appears not to affect the ability of F419 to promote the growth of cells in monolayer but does correlate with the impaired ability of F419 to promote anchorage-independent growth.

The study of ROS-induced signaling and transformation has been hampered by the absence of a known ligand for c-Ros and the inability to overexpress wild-type c-Ros in several types of mammalian cells. One strategy employed to overcome these obstacles involved the generation of two chimeric receptors, ER1 and ER2, between the epidermal growth factor receptor (EGFR) and c-Ros (8). The ER1 chimeric receptor contains the extracellular and transmembrane domains of EGFR fused to the cytoplasmic domain of c-Ros.
substrates involved in protein synthesis. Activated MAPK also translocates to the nucleus to phosphorylate transcription factors leading to activation of gene expression. Considering the high frequency of Ras mutations in cancer, the Ras-Raf-MEK-MAPK pathway has been linked to cell proliferation and cancer. However, no recurring mutations downstream of Ras in the MAPK pathway have been identified in human malignancies (11). In addition, MAPK has not been consistently shown to be required for survival, a key component in tumor development (12–14).

The PI3K signaling pathway has been shown to be essential for growth factor-mediated promotion of cell survival (14, 15). PI3K is a lipid kinase that is recruited to the membrane and activated by RPT kinase. At the plasma membrane, PI3K phosphorylates the phosphatidylinositols (PtdIns) to generate PtdIns (3)P, PtdIns (3,4)P2, or PtdIns (3,4,5)P3 (PIP3). These phosphorylated substrates then serve to recruit and activate a diverse array of proteins that contain pleckstrin homology domains that interact with the lipid moiety. The plethora of such proteins includes protein kinases (PDK1, PDK2, and Akt), nucleotide exchange factors (Tiam1, Vav, and Sas (son of sevenless) 1), GTPase-activating factors, phospholipases, and many others (16, 17). The serine/threonine protein kinase Akt is a major downstream effector of PI3K whose function contributes to both growth (18–21) and survival (12, 13, 22, 23), which are key requirements in tumor growth and progression. The list of Akt substrates comprises of at least 13 molecules. These substrates have been shown to be involved in growth (mTOR (mammalian target of rapamycin), p21, FKHR, and GSK-3β (glycogen synthase kinase-3β)), survival (FKHR, Bad, caspase-9, CREB, p21, IKK, and apoptosis signal-regulating kinase 1 (ASK1)), metabolism (GLUT4, phosphofructokinase-2, and GSK-3β), and possibly angiogenesis and DNA repair (eNOS (endothelial nitric oxide synthase) and BRCA1 (breast cancer susceptibility gene)) (11, 24, 25). The rapamycin-sensitive mammalian TOR (mTOR) regulates the ribosomal S6 kinase, p70S6K, which is a key regulator of cell growth through its phosphorylation of 40S ribosomal protein S6, a major component of the protein synthesis machinery (11).

As mentioned above, in addition to recruiting the survival factor Akt, PIP3 also recruits nucleotide exchange factors such as Tiam1 and Vav family members to the membrane, which subsequently leads to the activation of the Rho family of GTPases (Rac, Rho, and Cdc42). Under physiologic conditions, Rac, Rho, and Cdc42 regulate the actin cytoskeleton and help to maintain cell-cell contacts and cell-matrix interactions that are crucial for establishing the polarized epithelium. Rac regulates the formation of membrane ruffles called lamellipodia (26), Rho is involved in the formation of stress fibers (27), and Cdc42 regulates the formation of pole-like projections called filapodia (26). Under tumorigenic conditions, however, Rac, Rho, and Cdc42 have been implicated in motility and invasion (28). The first evidence implicating Rho family members in migration and invasion came from the identification of Tiam1 from a retroviral insertional mutagenesis screen (29). Tiam1 and its downstream effectors, Rac and Cdc42, were identified as the factors responsible for the invasion of the virus-induced T-cell lymphoma (29, 30). Rho has also been shown to promote invasion of tumor cells (27, 31–33). Ros has also been shown to recruit Vav3, another member of the Vav family of guanine nucleotide exchange factors, which can also regulate the Rho family GTPases (34).

In addition to the MAPK and the PI3K pathways, a previous study showed that STAT3 was also activated by Ros (9). Of the six STAT family members, STAT3 stands out in its involvement in growth and oncogenesis. STAT3 knock-out mice are
PI3K, Rho GTPases, and STAT3 in Ros-induced Transformation

In the present report, we explore the role of the MAPK and PI3K pathways in Ros-induced growth and transformation of CEF and NIH3T3 cells. Our results indicate that the MAPK pathway plays a minor role in the monolayer growth and no significant role in anchorage-independent growth of Ros-transformed cells. By contrast, the PI3K pathway plays a more significant role in the growth of Ros-transformed cells in monolayer and is essential for their anchorage-independent growth. We also found that overexpression of a constitutively activated STAT3 could alleviate the inhibitory effect of the PI3K drug inhibitor LY294002 on colony formation in soft agar. These results suggest that PI3K and STAT3 share certain overlapping functions in promoting anchorage-independent growth of Ros-transformed cells.

EXPERIMENTAL PROCEDURES

**Cells and Viruses**—CEF were prepared from 11-day-old embryos and maintained according to previously published protocols (6). CEF cells were maintained in monolayer in F-10 medium containing 5% calf serum and 1% chick serum. The molecularly cloned avian sarcoma viruses UR2, F419, and DI used in this study (Fig. 1A) have been described previously (4, 7, 38). Briefly, the F419 mutant contains a tyrosine to phenylalanine mutation at amino acid 419, and the DI mutant contains a deletion of the unique 6-amino acid insertion found in UR2 located 3 amino acids downstream of the predicted catalytic loop of the PT kinase domain. NIH3T3 cells were maintained in monolayer in the regular Eagle's medium (DMEW) with 10% calf serum.

**Plasmids**—The EGFR-Ros chimeric receptor constructs (ER1 and ER2) (Fig. 1C) have been described previously (8). The Δ85 construct was obtained from Dr. Julian Downdward (Signal Transduction Laboratory, Imperial Cancer Research Fund, London, U.K.). The PTEN construct was obtained from Dr. K. M. Yamada (National Institutes of Health, Bethesda, MD). The constitutively active myristoylated Akt (myrAkt) and dominant negative, kinase dead Akt (dnAkt) constructs were obtained from Dr. Phillip Tschissl (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA). The BD110 construct was obtained from Dr. Yasu Fukui (University of Tokyo, Tokyo, Japan). The dominant negative MEK (LIDA) was obtained from Dr. M. Weber (University of Virginia). The dominant negative ERK2 was obtain from M. Cobb (University of Texas Southwestern Medical Center). The original c-myc-tagged Cdc42 (Cdc42 Rac1Y12V, RhoAL63, Cdc42V12) and dnRac1Rho (Rac1N17, RhoAN19) constructs were obtained from Dr. Toru Miki (39). The dnCdc42 (Cdc42N17) and C3 transferase were obtained from Dr. Alan Hall (40). The original activated and dominant negative constructs were hemagglutinin-tagged and transferred to the pEF-neo vector as described previously (34). ACK24 was obtained from Dr. Maruta (41). STAT3C was obtained from Dr. James Darnell (42).

**Reagents and Antibodies**—Anti-phospho-Akt, anti-Akt, anti-phospho-p70S6 kinase, and anti-phospho-AMPK antibodies were purchased from Cell Signaling. Anti-ERK2 (C-14) and anti-p70S6 kinase antibodies were purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to horseradish peroxidase were purchased from Transduction Laboratories. LY294002 (2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one), PD98059, and rapamycin were purchased from Calbiochem. The LipofectAMINE 2000 reagent was purchased from Invitrogen.

**Growth Rate Assay**—Cells were grown in medium containing 4% serum in the presence or absence of the different drug inhibitors at varying concentrations. Cell numbers were counted on the 4th day. Day 0 was defined as the day of seeding. Day 1 was defined as the day when drug treatment started.

**Colony Formation Assay**—5 × 10^4 to 10^5 cells were resuspended with or without specific inhibitors (10 or 25 μM PD98059, 5 or 10 μM LY294002, 5 or 10 ng/ml rapamycin) in 4 ml of top agar medium (DMEW with 10% calf serum and 0.4% agar) and layered over 5 ml of bottom agar medium (DMEW with 10% calf serum and 0.7% agar) in 6-mm dishes. The medium, with or without specific inhibitor, was laid over the bilayered soft agar every 5 days. To analyze the effect of the dominant negative or constitutively active mutants of various signaling molecules on the colony-forming ability of control and transformed cells, an empty plasmid or mutant-expressing vector was transfected into the transformed cells using the LipofectAMINE 2000 reagent. In addition, to monitor the transfection efficiency, 1/10 of the amount of pGFP plasmid was also transfected into the transformed cells; alternatively, a parallel culture was transfected with an equivalent amount of pGFP plasmid. A subculture was observed under a fluorescence microscope 24–48 h after transfection to assess the transfection efficiency based on the expression of GFP. Typically, 50–70% transfection efficiency was obtained for NIH3T3 cells, and 50–55% efficiency was obtained for CEF. 48 h post-transfection, 5 × 10^4 to 10^6 cells were subjected to colony formation assay as described above. All of the colony assays for EGFR-Ros-expressing NIH3T3 cells were in the presence or absence (as control) of 30 ng/ml EGF. Cells not used for colony assay were extracted with radioimmunoprecipitation buffer, and total protein lysates were analyzed for expression of the dominant negative or constitutively active mutants. The colonies were stained on day 10–14 with iodonitrotetrazolium (0.25 mg/ml in H_2O or regular medium) for 2 days at 37 °C before counting.

**RESULTS**

**Ros-induced Anchorage-independent Growth of CEF and NIH3T3 Cells**—The UR2 virus and its mutants DI and F419 are described above. We have shown previously that all three viral encoded PT kinases are kinase-active and promote growth in CEF (7) (Fig. 1A). All three viruses are able to induce colony formation of infected CEF in soft agar; however, the transforming abilities of F419 and DI were attenuated compared with that of UR2 as reflected in the size of the induced CEF colonies (Fig. 1B). The EGFR-Ros chimeras (ER1 and ER2) have been described previously, and their sequence make-ups are depicted in Fig. 1C. ER1 and ER2 induce colony formation of NIH3T3 cells in soft agar only in the presence of EGF (8) (Fig. 1D).

**Effect of PD98059, LY294002, and Rapamycin on the Monolayer Growth of Normal and Ros-expressing CEF and NIH3T3 Cells**—The MAPK, PI3K, and p70S6K signaling pathways have been associated with growth factor receptor-mediated mitogenesis in various cell lines. We wanted to assess the significance of these pathways in monolayer growth of control and Ros-transformed CEF and NIH3T3 cells. The Ros-transformed cells were seeded at low density and permitted to proliferate in the presence or absence of various drug inhibitors in the regular maintenance medium. The drug inhibitors that were utilized included PD98059, which is a specific inhibitor of MEK in the MAPK pathway, LY294002, which is a specific inhibitor of PI3K, and rapamycin, which is a specific inhibitor of mTOR/FRAP that is required to activate p70S6K. PD98059, LY294002, and rapamycin were able to inhibit MAPK, Akt (as a measure of PI3K activity), and p70S6K, respectively, substantially as reflected by the decrease of the respective phosphorylated forms of the proteins at the two dosages tested (Fig. 2, A and B). In UR2 CEF, the degree of inhibition of Erk and Akt by 25 μM PD98059 and 10 μM LY294002 was similar compared with the untreated controls (Fig. 2A). In EGFR-Ros-expressing NIH3T3 cells, the upper species of the activated Erk was almost completely inhibited, and the lower species was inhibited more than 75% at 25 μM PD98059 (Fig. 2B). The pattern of growth inhibition by the three drugs was consistent among all control and transformed cells (Fig. 2, C and D). Overall, inhibiting the MAPK pathway with the MEK inhibitor PD98059 only slightly inhibited the monolayer growth of CEF infected by UR2 or the two UR2 mutants, F419 and DI, grew on average to 83 and 68% of their untreated counterparts in the presence of 10 μM and 25 μM PD98059, respectively. The control NIH3T3 cells (N5), along with those stably transfected with either of the two EGFR-Ros chimeric receptors (ER1 or ER2), in the presence of EGF grew to an average of 84% and 65%.
of the untreated controls in the presence of 10 \( \mu M \) and 25 \( \mu M \) PD98059, respectively. In contrast, inhibiting the PI3K pathway with LY294002 had a more substantial effect on the monolayer growth of normal and infected CEF and the control and ER1/ER2-expressing NIH3T3 cells, although CEF appeared to be more sensitive than the NIH3T3 cells to LY294002. The control and virus-infected CEF grew on average to only 45 and 27% of the untreated cells in the presence of 5 \( \mu M \) and 10 \( \mu M \) LY294002, respectively. Similarly, the control and ER1/ER2-expressing NIH3T3 cells grew to an average of 60 and 41% of the untreated counterparts in the presence of 5 \( \mu M \) and 10 \( \mu M \) LY294002, respectively. In CEF, inhibition of p70S6K with the anti-fungal and immunosuppressant agent rapamycin produced a moderate inhibition on monolayer growth which was slightly more significant than treatment with PD98059 but less dramatic than treatment with LY294002. Specifically, the control and virus-infected CEF grew to an average of 53 and 48% of the control cells when treated with 5 and 10 ng/ml rapamycin, respectively. The control and ER1/ER2-expressing NIH3T3 cells grew to an average of 77 and 70% of the untreated counterparts in the presence of 5 and 10 ng/ml rapamycin, respectively. Therefore, PI3K pathway is more important than MAPK in Ros-promoted monolayer growth of CEF and NIH3T3 cells.

**Effect of PD98059, LY294002, and Rapamycin on Colony Formation of Ros-expressing CEF and NIH3T3 Cells**

Inhibiting the MAPK pathway with PD98059 had a negligible effect on the colony-forming abilities of UR2, and its mutants infected CEF and of all NIH3T3 cells overexpressing the EGFR-Ros chimeric receptors ER1 and ER2 in the presence of EGF (Fig. 3). This lack of inhibition could not be accounted for by failure of the drug to inhibit the MAPK effectively (Fig. 2). The size and number of the colonies were unchanged in the presence of both concentrations of PD98059 (Fig. 3, A and B). In contrast, abrogating the PI3K pathway had a dramatic inhibi-
itory effect on colony formation of Ros-expressing cells, especially in NIH3T3 cells (Fig. 3, A and B). In CEF, the DI mutant-infected cells displayed a more significant inhibitory effect than UR2- and F419-infected cells in the presence of LY294002. Colony formation of DI-infected CEF was reduced to 21% in both concentrations of LY294002. On average, the colony-forming abilities of UR2- and F419-infected CEF were reduced to 55 and 37% of the controls in the presence of 5 and 10 \(\mu\)M LY294002, respectively (Fig. 3 C). Inhibition of p70S6K resulted in a differential effect on the UR2- versus the mutant-infected CEF. The F419- and DI-infected CEF were severely inhibited by rapamycin, decreasing their colony-forming abilities to an average of 31 and 26% of the control in the presence of 5 and 10 ng/ml rapamycin, respectively. In contrast, UR2-infected CEF only reduced their colony-forming ability to 83% of the control in both concentrations of rapamycin. The pattern of inhibition in the ER1 (clones 2 and 4) and ER2 (clone 69) clones were similar. On average, colony formation was reduced to 31 and 12% of the untreated controls in the presence of 5 and 10 \(\mu\)M LY294002, respectively (Fig. 3D). Inhibition of p70S6K in the NIH3T3 cells yielded a moderate inhibitory effect on the colony-forming ability of ER1/ER2-expressing cells. The effect was less severe than that of PI3K inhibition. The ER2 clone 69 appeared to be more sensitive to rapamycin than the ER1 clones 2 and 4. We conclude that MAPK signaling is not required, whereas PI3 kinase signaling is essential for Ros-induced anchorage-independent growth. Transformation of CEF by UR2 Ros, but not by its mutants, appears to abrogate some requirement of p70S6 kinase function for promoting anchorage-independent growth.

**Effect of Dominant Negative and Activated Mutants of the PI3K and MAPK Signaling Pathway on Ros-induced Anchorage-independent Growth**

To confirm further the role of PI3K signaling in the Ros-induced anchorage-independent growth as suggested by inhibition of the colony-forming abilities by the pharmacological drug LY294002, a genetic approach was employed by expressing dominant negative or constitutive activated mutants of the PI3K, Rho GTPases, and STAT3. An overlay of DMEM containing 10% calf serum with or without the appropriate concentration of drug inhibitors was added every 5 days for up to 21 days. The colonies were stained with 0.25 mg/ml p-iodonitrotetrazonium in normal growth medium for 2 days and counted. Panels A and B, representative photomicrograph of UR2-induced (panel A) or ER1–2-induced (panel B) colonies in the absence or presence of the drug inhibitors. Panels C and D, quantitative representation of the colony-forming abilities of the F419–, DI–, and UR2-infected CEF (panel C) or ER1–2, ER1–4, and ER2–69 EGFR-Ros chimera expressing NIH3T3 clones in the presence of EGF and with or without the indicated drug inhibitors (panel D). The data are presented as a percentage of colonies formed under drug treatment relative to untreated corresponding controls. The results are derived from three independent experiments.
expression of dnp85 and dnakt in ER1
Colonies were stained and counted after 14 days. The results were compiled and calculated from three independent experiments. Protein
ER1- or ER2-expressing NIH3T3 cells were transfected with 4
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MAPK on colony formation of CEF and NIH3T3 cells expressing Ros or its mutants. Normal CEF, UR2, F419, or DI-infected CEF or
ER1- or ER2-expressing NIH3T3 cells were transfected with 4 μg of pGFP, empty vector, or vector expressing constitutively active PI3K (BD110),
dnPI3K (dnp85), PTEN, dnakt, dnMEK, or dnERK2. pGFP was used to assess the transfection efficiency 48 h after transfection by observation under a fluorescence microscope. The percentage of CEF that expressed GFP ranged from 25 to 50%, whereas that for NIH3T3 cells ranged from 50 to 70%. Colony assays were set up 48 h after transfection. Panel A, UR2-infected CEF were transfected with control or vector expressing dnp85, PTEN, or dnakt. Results were compiled and calculated from four independent experiments. The right images represent protein expression of dnp85, PTEN, and dnakt in transfected CEF. Panel B, normal CEF were transfected with BD110 or myrAkt vector. F419- and DI-infected CEF were transfected with control or vector expressing a BD110 or myrAkt. Colonies were stained and counted after 14 days. Results were compiled and calculated from four independent experiments. Protein expression of BD110 and myrAkt in F419- and DI-infected CEF is shown on the right. Panel C, NIH3T3 cell clones expressing the EGFR-Ros chimeric receptors ER1 or ER2 were transfected with 4 μg of control, dnp85, or dnakt vector. Colonies were stained and counted after 14 days. The results were compiled and calculated from three independent experiments. Protein expression of dnp85 and dnakt in ER1-2- and ER2-69-expressing NIH3T3 cells is shown on the right. Panel D, ER1 cells were transfected with control, dnMEK, or dnERK2. Histograms of vector and dnMEK represent three independent experiments, and that of dnERK2 represents a single experiment. Protein expression of dnMEK and dnERK2 is shown on the right.

transfection. Overexpression of the dominant negative PI3K (ap85) or the PI3K phosphatase PTEN in UR2-infected CEF resulted in a decrease in colony formation to 44 and 41% of the control, respectively (Fig. 4A). When UR2 CEF were transfected with dnp85 and GFP vector at a ratio of 10:1 and subsequently sorted for GFP expression, the colony-forming ability of the GFP-positive cells was reduced by 80% compared with control in repeated experiments, suggesting that most of the dnp85-expressing UR2 were inhibited for colony formation (data not shown). Overexpression of dnakt, the major downstream effector of PI3K, reduced the colony-forming ability of UR2-infected CEF to 45% of the control (Fig. 4A). By contrast, expression of a dominant negative mutant of MEK or MAPK in EGFR-Ros (ER1-2)-expressing NIH3T3 did not result in significant inhibition of their colony-forming ability (Fig. 4D). The UR2 mutants F419 and DI could still transform CEF and induce colony formation, although both were attenuated in their colony-forming abilities compared with UR2 (Fig. 1B). We next tested to see whether activation of the PI3K-Akt pathway could rescue the attenuated colony-promoting ability of the two UR2 mutants. The constitutively active mutants of PI3K (BD110) and Akt (myrAkt) were used (Fig. 4B). Expression of BD110 or myrAkt alone in CEF did not result in colony formation under our conditions (Fig. 4B). However, expression of BD110 in F419- and

DI-infected CEF resulted in an increase in colony-forming ability to 143 and 168% of the controls, respectively (Fig. 4B). The myrAkt could also increase the colony-forming ability of F419- and DI-infected CEF to 182 and 164% of the controls, respectively (Fig. 4B). Similarly, the ap85 mutant reduced the colony-forming ability of the ER1 and ER2 cells to 50 and 58% of the controls, respectively (Fig. 4C). In addition, the dnakt decreased the colony-forming ability of the ER1 and ER2-overexpressing NIH3T3 cells to 54 and 52% of the control, respectively (Fig. 4C).

Role of Rho Family GTPases in Ros-induced Transformation—We have shown previously that Ros is able to interact and tyrosine phosphorylate the guanine nucleotide exchange factor Vav3, which is capable of activating Rac, Rho, and Cdc42 (34). In addition, Ros can activate PI3K, which in turn can lead to the recruitment and activation of guanine nucleotide exchange factors such as Tiam1 (29). Similarly, Tiam1 then can activate the Rho family of small GTPases, including Rac, Rho, and Cdc42, which are known to be involved in cytoskeletal rearrangement, migration, and invasion as mentioned above (28). We assessed the significance of the Rho family GTPases in Ros-induced transformation by overexpressing the dominant negative mutants of these proteins in the Ros-expressing cells (Figs. 5B and 6C). Coexpression of dnRac (Rac1N17) in UR2-infected CEF decreased the colony-forming ability to 51% of the
control (Fig. 5A). Coexpressing a dominant negative Rho (RhoAN19) or the exoenzyme C3 transferase, an ADP-ribosyl transferase that selectively ribosylates Rho proteins on asparagine residue 41 to block the Rho activity (40) decreased the colony-forming ability of UR2-infected CEF to 45 and 44% of the control, respectively (Fig. 5A). The dominant negative Cdc42 (Cdc42N17) reduced the colony formation in UR2-infected CEF to 47% of the control (Fig. 5A). Furthermore, ACK42, a 42-amino acid small GTPase-binding peptide derived from ACK, a downstream effector of Cdc42 (41), decreased the colony-forming ability of UR2-infected CEF to 54% (Fig. 5A).

In NIH3T3 cells, Rac seems to play a less significant role in anchorage-independent growth. Coexpression of dnRac in ER1/ER2-expressing NIH3T3 cells reduced the colony-forming ability to 72 and 90% of the controls, respectively (Fig. 6, A and B). Inhibiting Rho and Cdc42 functions in the ER1/ER2-expressing NIH3T3 cells had a dramatic inhibitory effect similar to that seen in the UR2-infected CEF. Coexpression of dnRho in ER1/ER2-expressing NIH3T3 cells reduced the colony-forming ability to 51 and 57% of the controls, respectively (Fig. 6, A and B). In addition, coexpression of C3 transferase in ER1/ER2-expressing NIH3T3 cells reduced the colony-forming ability to 43 and 47% of the controls, respectively (Fig. 6, A and B). Coexpression of dnCdc42 in ER1/ER2-expressing NIH3T3 cells reduced the colony-forming ability to 43 and 59%, and coexpression of ACK reduced the colony-forming ability to 62 and 61% of the controls, respectively (Fig. 6, A and B). Our results show that signaling of Rho family GTPases play an important role in Ros-promoted anchorage-independent growth. However, Rac1 appears to have only a minor role in Ros-induced anchorage-independent growth of NIH3T3 cells.

STAT3 and PI3K Share Overlapping Function in Ros-induced Transformation—We have shown previously that STAT3 was activated by Ros, and this activation was required for Ros-induced cell transformation in the EGFR-Ros (ER2) chimeric receptor-expressing NIH3T3 cells (9). Ros can also activate PI3K (38), and the data described above suggest that the PI3K pathway is essential for Ros-induced anchorage-independent growth. Because signaling through both STAT3 and PI3K is essential for Ros-induced cell transformation, we questioned whether the two signaling pathways are completely independent or share overlapping function in mediating the cell transformation. If the two pathways share overlapping functions, we reasoned that overexpression of a constitutively active mutant of STAT3 (STAT3C) (Fig. 7C) was able to rescue the colony-forming ability of ER1 and ER2 in the

Fig. 5. Effect of dominant negative mutants of Rho family GTPases on colony formation of UR2-transformed CEF. Panel A, UR2-infected CEF were transfected with 4 μg of pC3FP, control vector, or vector expressing dnRac (RacN17), dnRho (RhoAN19), C3 transferase, dnCdc42 (Cdc42N17), or ACK42. Colony assays were as described under “Experimental Procedures” and in the Fig. 4 legend. Colonies were stained and counted after 14–21 days. Panel B, expression of the dnRac/Rho/Cdc42 mutants in the transfected CEF. Results were compiled and calculated from three independent experiments. IB, immunoblot.

Fig. 6. Effect of dominant negative mutants of Rho family GTPases on EGFR-Ros-induced colony formation of NIH3T3 cells in response to EGF. Panels A and B, ER1 and ER2 NIH3T3 cells were transfected with 4 μg of pC3FP, control vector, or vector expressing dnRac (RacN17), dnRho (RhoAN19), C3 transferase, dnCdc42 (Cdc42N17), or ACK42. Colony assays were as described under “Experimental Procedures” and in the Fig. 4 legend. Colonies were stained and counted after 14–21 days. Panel C, expression of the dnRac/Rho/Cdc42 mutants in the transfected NIH3T3 cells. Results were compiled and calculated from three independent experiments.
transformation of the cells (8, 9, 34). We have also shown that the two UR2 mutants, F419 and DI, retain wild-type protein-tyrosine kinase and mitogenic activities in infected CEF maintained in monolayer culture but display dramatically reduced ability to promote anchorage-independent growth (Fig. 1A) and tumor formation in chickens (38). To dissect further the downstream components of Ros signaling which are important for transformation, we have employed the inducible Ros chimera and the loss-of-function mutants of Ros in NIH3T3 and CEF systems to investigate the effect of various chemical inhibitors as well as genetic inhibitors and activators of specific signaling pathways on Ros-induced transformation. Our results indicate that PI3 kinase and its downstream signaling cascades play an essential role in the transformation of CEF and NIH3T3 cells by Ros. This conclusion is supported by the experimental evidence through the use of specific PI3K drug inhibitor LY294002 (Fig. 3), a dominant negative version of PI3K (Δp85) (Fig. 4, A and C), and a PI3 phosphatase antagonist of PI3K, PTEN (Fig. 4A). Our results of the effect of a dominant negative mutant of Akt on Ros-induced cell transformation indicate that the Akt branch of the PI3K signaling cascade is important for anchorage-independent growth of CEF and NIH3T3 cells induced by Ros (Fig. 4, A and C). Furthermore, overexpression of the constitutively activated PI3K (BD110) or Akt (myrAkt), was able to increase the colony-forming abilities of the transfection-attenuated mutants F419 and DI substantially (Fig. 4B). This result suggests that failure to activate PI3K signaling in CEF when deprived of anchorage to substratum by these Ros mutants is most likely, at least in part, responsible for their defectiveness in promoting anchorage-independent growth and tumor formation. Among the RPT kinases, Ros PT kinase domain shares the greatest homology with that of the insulin receptor and IGFR, suggesting that they may possess common downstream signaling pathways and effectors. The current results are consistent with their previous findings that MAPK was either only partially required (45) or indispensable (46) for an oncogenic IGFR-induced cell transformation, depending upon the cell type, whereas PI3K was absolutely required for transformation induced by the oncogenic forms of insulin receptor and IGFR, although it was not important for v-Src-induced anchorage-independent growth (46).

The Rho family GTPases (Rac, Rho, and Cdc42) have been shown to be required for Ras-induced transformation (41, 47–49). Rho has also been shown to be required for transformation induced by an oncogenic epidermal growth factor receptor (50). In the present study, we also show that the Rac, RhoA, and Cdc42 are required for the RPT kinase Ros-induced anchorage-independent growth of CEF and NIH3T3 cells, although Rac may contribute more in chicken cells than in mammalian cells because dnRac affected UR2-infected CEF colony formation more severely than that of ER1- and ER2-overexpressing NIH3T3 cells (Figs. 5A and 6, A and B). This is in agreement with our previous observations that dnRac could only inhibit the oncogenic IGFR-induced colony formation of NIH3T3 cells by 40% (45). Our results suggest that Rac signaling is not as important as that of Rho and Cdc42 for anchorage-independent growth of NIH3T3 cells. At present it is unclear whether the activation of the Rho family GTPases by Ros is via a PI3K-dependent and/or -independent pathway. We have shown previously that Ros can recruit and activate Vav3, a guanine nucleotide exchange factor, which can directly activate the Rho family GTPases (34). Our current data suggest that Vav3 or its related guanine exchange factors may play an important role in Ros-induced cell transformation.

Another signaling pathway activated by Ros is that of the STAT, specifically STAT3. Our previous observation demon-
PI3K, Rho GTPases, and STAT3 in Ros-induced Transformation

...also essential for EGFR-Ros-induced colony formation. Therefore, it appears that the activation of the endogenous level of either STAT3 or PI3K alone by Ros is not sufficient to compensate for the loss of the other by chemical or genetic inhibitor. However, we show here that boosting the signaling activity of one (STAT3) could then compensate partly for the loss of the other (PI3K). The converse may also be true, although this remains to be tested. This result suggests that STAT3 and PI3K signaling pathways may share certain overlapping function in mediating Ros-induced anchorage-independent growth. Previous studies have shown that overexpression of STAT3 could affect levels and activity of p21\(^{Waf1}\), which would have a direct consequence on the progression of the cell cycle and on apoptosis (51, 52). Recently, Zhou et al. (53) showed that Akt can phosphorylate p21\(^{Waf1}\) resulting in its translocation from the nucleus to the cytoplasm. The effect is the loss of growth inhibition because of a lack of nuclear p21 and the association between cytoplasmic p21 and the apoptosis signal-regulating kinase 1 (ASK1) leading to the resistance to apoptosis. It is possible that Ros-activated PI3K and STAT3 can each lead to regulation of the cell cycle check point proteins, and this may represent the overlapping function of the two signaling pathways.

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