Matrix-independent Activation of Phosphatidylinositol 3-Kinase, Stat3, and Cyclin A-associated Cdk2 Is Essential for Anchorage-independent Growth of v-Ros-transformed Chicken Embryo Fibroblasts*

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Shailaja Uttamsingh, Cong S. Zong, and Lu-Hai Wang‡
From the Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

The question remains open whether the signaling pathways shown to be important for growth and transformation in adherent cultures proceed similarly and play similar roles for cells grown under anchorage-independent conditions. Chicken embryo fibroblasts (CEF) infected with the avian sarcoma virus UR2, encoding the oncogenic receptor protein-tyrosine kinase (RPTK) v-Ros, or with two of its transformation-impaired mutants were grown in nonadherent conditions in methylcellulose (MC)-containing medium, and the signaling functions essential for Ros-induced anchorage-independent growth were analyzed. We found that the overall tyrosine phosphorylation of cellular proteins in CEF transformed by v-Ros or by two oncogenic nonreceptor protein-tyrosine kinases (PTKs), v-Src and v-Yes, was dramatically reduced in nonadherent conditions compared with that in adherent conditions, indicating that cell adhesion to the extracellular matrix plays an important role in efficient substrate phosphorylation by these constitutively activated PTKs. The UR2 transformation-defective mutants were differentially impaired compared with UR2 in the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and Stat3 in nonadherent conditions. Consistently, the constitutively activated mutants of PI 3-kinase and Stat3 rescued the ability of the UR2 mutants to promote anchorage-independent growth. Conversely, dominant negative mutants of PI 3-kinase and Stat3 inhibited UR2-induced anchorage-independent growth. UR2-infected CEF grown in nonadherent conditions displayed faster cell cycle progression than the control or the UR2 mutant-infected cells, and this appeared to correlate with a PI 3-kinase-dependent increase in cyclin A-associated Cdk2 activity. Treatment of UR2-infected cells with Cdk2 inhibitors led to the loss of the anchorage-independent growth-promoting activity of UR2. In conclusion, we have adopted an experimental system enabling us to study the signaling pathways in cells grown under anchorage-independent conditions and have identified matrix-independent activation of PI 3-kinase and Stat3 signaling functions, as well as the PI 3-kinase-dependent increase of cyclin A-associated Cdk2 kinase activity, to be critical for the Ros-PTK-induced anchorage-independent growth.

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‡ To whom correspondence should be addressed: Dept. of Microbiology, Box 1124, Mount Sinai School of Medicine, New York, NY 10029. Tel.: 212-241-3796; Fax: 212-334-1684; E-mail: lu-hai.wang@mssm.edu.

The proto-oncogene c-ros codes for a receptor protein-tyrosine kinase with an extended extracellular (EC) domain (1–3). The normal function and the ligand of c-Ros remain elusive. The temporal and tissue expression profile of c-Ros in chicken and rats suggests that it plays a role in epithelial cell differentiation during embryogenesis, particularly in the tubules of the kidney and testis (4–7).

The v-rosv oncogene of the avian sarcoma virus UR2 encodes a constitutively active 68-kDa RPTK, derived by the fusion of viral gag to the N-terminal truncated c-Ros, which is expressed as a transmembrane protein with the gag moiety protruding extracellularly (8–11). To explore the functional role of specific tyrosine residues of v-Ros, particularly with respect to their roles in mitogenic versus transforming ability, we had generated and analyzed a series of loss-of-function mutants (12). The two mutants F419 and DI (Fig. 1A) displayed wild type kinase and mitogenic activities of CEF grown in monolayer but were attenuated in their ability to promote anchorage-independent growth. F419 contains a phenylalanine substitution for the third tyrosine (Y419F) in the triple tyrosine cluster within the Ros PTK domain. DI harbors a 6-amino acid deletion 3 amino acids downstream of the predicted catalytic loop of the Ros PTK. These mutants proved to be invaluable tools for the identification of signaling pathways essential for anchorage-independent growth. Employing CEF grown in monolayer, we found that both F419 and DI displayed a reduced ability to induce the tyrosine phosphorylation of a series of cytoskeletal proteins involved in cell-cell and cell-matrix interactions (12). Additionally, F419 was impaired in the tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) and in inducing IRS-1-associated PI 3-kinase activity (12).

The ability of cells to proliferate in the absence of adhesion to the extracellular matrix (ECM) or anchorage-independent growth is one of the hallmarks of transformed cells and is also the best in vitro correlate of tumorigenicity (13). It is thus likely that the molecular mechanisms facilitating anchorage-independent growth in vitro are also responsible for the aggressive growth properties of naturally occurring tumors. The signaling pathways activated by the binding of the cognate growth factors to RPTKs on the cell surface and by the integrin-mediated adhesion to the extracellular matrix (ECM) jointly regulate cell

1 The abbreviations used are: EC, extracellular; RPTK, receptor protein-tyrosine kinase; PTK, protein-tyrosine kinase; IRS-1, insulin receptor substrate 1; PI 3-kinase, phosphatidylinositol 3-kinase; ECM, extracellular matrix; STAT, signal transducer and activator of transcription; Rb, retinoblastoma; CEF, chicken embryo fibroblasts(s); dn, dominant negative, ca, constitutively activated; GFP, green fluorescent protein; MAP, mitogen-activated protein; Ab, antibody; MBP, myelin basic protein; PLCγ, phospholipase Cγ; FAK, focal adhesion kinase.
cycle progression (reviewed in Refs. 14–17), and both arms of signaling are required for the growth of normal cells. The cytoplasmic signaling pathways include protein-tyrosine and serine/threonine kinase cascades, small GTPases, inositol lipid pathways, and other phospholipid cascades (reviewed in Refs. 18–21). When cells are dissociated from the ECM, attenuating the activation of one or more of these pathways, fibroblasts typically undergo cell cycle arrest (22), whereas epithelial (23) and endothelial (24) cells undergo anchorage-related apoptosis or “anokias.” In transformed cells, alterations and activation of certain signaling functions including integrin-associated signaling may presumably abrogate the requirement for adhesion to the ECM, leading to increased cell cycle progression in fibroblasts or anokias resistance in epithelial and endothelial cells, thereby facilitating their anchorage-independent growth.

Phosphatidylinositol 3-kinase is a lipid and protein kinase activated by RPTKs. Upon activation, PI 3-kinase phosphorylates inositol lipids at the D-3 position of the inositol ring to generate 3-phosphatidylinositol phosphates such as phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. These phosphatidylinositol phosphates then function as second messengers serving as binding sites for proteins containing pleckstrin homology domains or FYVE fingers (25). PI 3-kinase has key regulatory functions in many cellular processes such as cell proliferation (26), differentiation (27, 28), apoptosis (29), sugar metabolism (30, 31), and vesicle trafficking (32). It has been shown that activation of PI 3-kinase is sufficient for cell cycle entry, through activation of Cdk4 and Cdk2, and induction of DNA synthesis (33). The signal transducers and activators of transcription (STATs) are activated by tyrosine phosphorylation, resulting in their dimerization and translocation to the nucleus, where they regulate the transcription of target genes by binding to specific DNA-response elements. STATs are known to function as effectors for a variety of growth factor receptors (34–38). STATs have been found to be activated in a wide variety of tumors and oncogene-transformed cell lines (reviewed in Ref. 39). Stat3 was shown to be constitutively activated in src-transformed NIH3T3 cells (40, 41), and its activation was essential for src-induced cell transformation (42, 43). We have also previously reported that Stat3 plays an important role in oncogenic Ros- and insulin-like growth factor-1 receptor-induced transformation of NIH3T3 cells, particularly in their anchorage-independent growth (44).

Anchorage may modulate cell cycle progression in fibroblasts by regulating various components of the G1 phase. The G1 to S phase transition in the cell cycle is controlled by functionally distinct Cdks that are sequentially regulated by cyclins D1–3, E, and A. The D cyclins associate with Cdk4 and Cdk6, whereas cyclins E and A associate with Cdk2. In the absence of ECM adhesion, the transcription and translation of cyclin D1 are blocked in primary human fibroblasts and some fibroblast cell lines (45, 46), and the growth-factor-dependent induction of cyclin E-associated Cdk2 activity fails to occur. The latter may be due to an increase in the steady state levels of the cip/kip family (p21\textsuperscript{WAF1/CIP1}, p27\textsuperscript{KIP1}, and p57) of cyclin-dependent kinase inhibitors, as well as redistribution of the total p21/p27 pool from the cyclin D1-Cdk4/6 to the cyclin E-Cdk2 complexes as a consequence of reduced adhesion-dependent expression of cyclin D1 (46). Since cyclin D-Cdk4/6-mediated phosphorylation disrupts retinoblastoma (Rb)-E2F and p107-E2F complexes, ECM adhesion also controls the induction of E2F target genes such as cyclin E and A (45–48). E2F-independent mechanisms for the expression of cyclin A have also been described (47, 49). Nonadherent v-Ras-infected ER-1–2 cells, which are transformation revertants, show mitogen-induced cyclin D1 expression, cyclin E-Cdk2 activity, and Rb phosphorylation, but not cyclin A expression, and the ectopic expression of cyclin A in these cells can rescue the block of S phase entry (47).

The vast majority of the studies on signaling pathways involved in cell transformation, including our studies with UR2 and its mutants so far, have employed cells grown in adherent cultures. Only very recently has there been some information available about signaling in nonadherent conditions. Whereas these limited studies have contributed to our understanding of the role of ECM adhesion in cellular signal transduction, a great deal still needs to be learnt about the signaling pathways that enable transformed cells to grow in an anchorage-independent manner. We have used a system that permits the growth and colony formation of cells in nonadherent conditions and the subsequent recovery of the cells, such that the signal transduction pathways that are operative in the absence of adhesion and are required for anchorage-independent growth of the transformed cells can be characterized. In order to identify the molecular mechanisms underlying UR2-mediated anchorage-independent growth, the signaling pathways activated by UR2 and its mutants, F419 and DI, have been analyzed and compared in adherent and nonadherent conditions. In anchorage-independent conditions, UR2-expressing cells displayed a faster cell cycle progression than the control and the mutant-expressing CEF. UR2, but not its mutants, was able to induce the matrix-independent activation of IRS-1-associated PI 3-kinase, the tyrosine phosphorylation of Stat3, and the increase of cyclin A-associated Cdk2 activity, these signaling pathways were found to be critical for v-Ros-induced anchorage-independent growth.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Chicken embryo fibroblasts (CEF) were prepared from 11-day embryos and maintained according to the published procedure (50). CEF were maintained in F-10/Dulbecco’s modified Eagle’s medium (1:1) supplemented with 5% calf serum and 1% chicken serum. Molecularly cloned avian sarcoma virus UR2 and its helper virus UR2AV have been described (10). The plasmids of UR2 and its mutants F419 and DI have been described (12). Virus stocks were obtained 10 days after DNA transfection with the respective viral DNA plasmids (see below) by collecting the overnight culture supernatants from the transfected cells.

**Plasmids**—The dpn85 construct was obtained from Dr. Julian Doward (Signal Transduction Laboratory, Imperial Cancer Research Fund, London, UK). The BD110 construct was obtained from Dr. Yasuhisa Fukui (University of Tokyo). Dominant negative (dn) Stat3–2 and constitutively activated (ca) Stat3-C were obtained from Dr. James Darnell (Rockefeller University). Us9-GFP was obtained from Dr. Andrew J. Beavis (Princeton University).

**DNA Transfections**—1 × 10\textsuperscript{5} CEF were plated per 60-mm dish 1 day before transfection. 20 μg of the UR2 or the F419/DI mutant plasmid and 2 μg of SacI-digested pUR2AV helper viral DNA were mixed and added to cells along with 30 μg/ml polybrene. The cells were incubated at 37 °C for 5–6 h, shocked with medium containing 25% MeSO\textsubscript{4}, washed, and replaced with regular growth medium.

Transfections of dn and ca plasmids were performed using the LipofectAMiner (2000 reagent (Invitrogen) according to the manufacturer’s protocols. 5 × 10\textsuperscript{5} CEF/100-mm dish were transfected with 25 μg of the empty vector or mutant-expressing vector along with one-tenth molar amount of the Us9-GFP plasmid. GFP-expressing cells were isolated by cell sorting using a MoFlo Cell Sorter (Cytometry, Fort Collins, CO).

**Biological Assays**—Cell transformation was monitored by morphological changes and anchorage-independent growth as described previously (51–53). Soft agar colony formation assays were carried out for 7–14 days, following which the colonies were stained with p-iodonitrotetrazolium (0.25 mg/ml) in normal growth medium for 2 days and counted. For colony formation in methylcellulose, medium containing 1.3% methylcellulose was used for the top layer instead of the 0.41% soft agar medium on the same platform of bottom layer agar.

**Culture and Recovery of Adherent and Nonadherent CEF**—Preparative methylcellulose culture conditions and recovery of cells from such cultures were according to the published procedures (54). Upon transformation, the transfected cells were amplified and either plated at a
density of $1 \times 10^6$ cells/100-mm dish or seeded at a density of $3 \times 10^6$ cells/75 ml of 1.3% methylcellulose-containing medium in 250-ml centrifuge tubes (Corning). After 48 h of incubation at 37 °C, the cells were treated with 200 μg Na$_2$VO$_4$ for 4 h. The methylcellulose cultures were diluted by the addition of 3 volumes of ice-cold Tris-Glu buffer to reduce the viscosity of the methylcellulose, and the cells were recovered by gentle centrifugation at 4 °C. The cells were further washed twice with the Tris-Glu buffer and recovered.

**Protein Analysis**—Protein extraction, immunoprecipitation, SDS-PAGE, and Western blotting have been described (8, 52).

**In vitro Kinase Assays**—In vitro assays of PTK, MAP kinase, and PI 3-kinase have also been previously described (53, 55). Cyclin D1-associated Cdk4/6 kinase assay and cyclin E- and cyclin A-associated Cdk2 assays followed the published procedures (56, 57).

**Antibodies and Inhibitors**—Anti-Ros and anti-IRS-1 antibodies were prepared in our laboratory and have been previously described (8, 11). All other antibodies were purchased from the following companies: PLCγ, p85, and FAK (for immunoprecipitation) antibodies from Upstate Biotechnology, Inc.; She, FAK (for Western blotting), paxillin, p130, Crk, β-catenin, γ-catenin, phosphotyrosine-horseradish peroxidase (RC20-HRP), mouse IgG-horseradish peroxidase, and rabbit IgG-horseradish peroxidase antibodies from Transduction Laboratories; Erk2, Cdk2, Cdk4, Cdk6, cyclin A, cyclin D1, cyclin E, p21, and p27 antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-FLAG Ab from Sigma; anti-retinoblastoma (Rb) Ab from Pharmingen; anti-pRb Ab from Cell Signaling; and anti-Myc Ab from the Mount Sinai Hybridoma Core Facility. All antibodies were used according to the manufacturer’s recommended dilutions. LY294002 and roscovitine were purchased from Calbiochem. Olomoucine was purchased from Sigma.

**Cell Cycle Analysis**—Cells plated in monolayer or cultured in methylcellulose medium were recovered after 48 h and fixed at 4 °C with 70% ethanol for 2 h. After the cells were washed with PBS, they were gently resuspended in 1 ml of DNA staining solution (PBS, pH 7.4, 1 μg/ml RNase A, 20 μg/ml propidium iodide). The cells were stored in the dark at 4 °C prior to flow cytometry analysis at the Mount Sinai Flow Cytometry Core Facility. Analysis was performed using Cell Quest software.

**RESULTS**

**Anchorage-independent Growth in Methylcellulose**—CEF infected with UR2 or its transformation-impaired mutants, F419 and DI, were grown under adherent (or anchorage-dependent) conditions in monolayer cultures or under nonadherent (anchorage-independent) conditions in methylcellulose cultures. In monolayer, UR2-infected CEF are highly transformed, displaying a characteristic elongated, fusiform, and refractile morphology (12). The colony forming ability of UR2-expressing CEF in methylcellulose essentially emulated that in soft agar; a large number of colonies were visible within 7–10 days (Fig. 1B). The F419 and DI mutant-expressing CEF showed dramatically reduced transforming abilities as reflected in the morphology, which resembled control cells (not shown), and in their diminished ability to form colonies in methylcellulose cultures (Fig. 1B). UR2-expressing CEF and UR2 mutant-expressing CEF were recovered from methylcellulose cultures at 2 and 4 days after seeding and counted to determine their growth rates (Fig. 1C). On day 2, the number of UR2-expressing CEF was more than double that of control CEF. The F419- and DI-expressing cells were differentially impaired in anchorage-independent growth compared with the UR2-expressing cells. By day 4, the reduced anchorage-independent growth of the control and the mutant-expressing CEF became even more apparent. In order to determine whether UR2 was promoting anchorage-independent growth by preventing cell death or by promoting cell cycle progression in nonadherent conditions, we performed a trypan blue exclusion test and cell cycle analysis. The trypan blue exclusion test revealed that the rate of cell death of the control CEF as well as the UR2- and mutant-expressing CEF was comparable, up to 10% in 2 days and up to 20% in 4 days of culturing the cells in MC medium (data not...
The kinase activity of UR2 and its mutants, F419 and DI, derived from CEF grown in adherent and nonadherent conditions was compared by assaying the intracellular tyrosine phosphorylation (Fig. 2A) and in vitro autophosphorylation (Fig. 2B) of the Ros protein. The relative abundance of UR2 and the mutant F419 and DI proteins were similar in CEF cultured in both conditions. Both mutants showed wild-type kinase activity intracellularly as reflected in their intracellular tyrosine phosphorylation, confirming our previous report (12). The intracellular phosphorylation of UR2 and the mutant proteins from CEF in nonadherent conditions was reduced less than 2-fold compared with that in adherent conditions (Fig. 2A). The amount of Ros protein immunoprecipitated from equivalent amounts of lysates obtained from cells grown in nonadherent conditions was slightly less compared with that in adherent conditions. Thus, the reduced phosphorylation of Ros in nonadherent conditions could be a reflection of less protein obtained in these conditions. However, specific tyrosine phosphorylation activity appeared not to be altered in the nonadherent conditions, reflecting equivalent specific PTK activity in adherent and nonadherent conditions. As expected, the DI protein migrated slightly faster than those of UR2 and F419 due to the 6-amino acid deletion. The in vitro kinase activity of UR2 and the mutant proteins derived from cells grown in nonadherent conditions was also reduced about 2-fold compared with that of the corresponding proteins from cells grown in adherent conditions (Fig. 2B). The greatly diminished in vitro kinase activity of the DI protein has previously been observed and is most likely due to its protein lability and inactivation in the process of extraction and subsequent in vitro processing for the assay as reported before (12). In adherent conditions, the tyrosine phosphorylation of cellular proteins (Fig. 2C) in UR2-expressing CEF was greatly increased over that in control cells. Both mutants showed reduced ability to phosphorylate cellular substrates. The reduction was more pronounced with F419. Surprisingly, the tyrosine phosphorylation of cellular substrates by UR2 and the mutant Ros PTKs in nonadherent conditions was dramatically reduced compared with that in adherent conditions despite comparable PTK activity of the Ros proteins in both conditions. Similar results were obtained with increased vanadate treatment of up to 600 μM in the MC medium, excluding the possibility that increased tyrosine phosphatase activity in the cells in MC medium may be responsible for the observed phenomenon (data not shown). This result suggests that adhesion to the ECM plays an important role in the ability of Ros PTK to phosphorylate its cellular substrates. Adhesion to the ECM was also required for efficient substrate phosphorylation by nonreceptor PTKs, since v-Src- or v-Yes-transformed CEF showed decreased tyrosine phosphorylation of cellular substrates in MC medium compared with that in monolayer (Fig. 2D).

MAP Kinase Activity—UR2 and its mutants, F419 and DI, were compared for their ability to phosphorylate and activate various signaling molecules in adherent and nonadherent conditions. The Ras/MAP (mitogen-activated protein) kinase pathway can be activated by RPTKs through Shc-Grb2-Sos and IRS-1-Grb2-Sos complex formation (58–62). The ability of UR2 and the mutant PTKs to activate MAP kinase was measured using myelin basic protein (MBP) as an exogenous substrate (Fig. 3A). We have previously observed an approximately 2-fold increase in MAP kinase activation in UR2-expressing cells and its mutant-expressing cells over the basal levels in control cells (12). However, no significant increase in MAP kinase activation was seen in the UR2- and mutant-expressing cells compared with the control CEF in the present experiments. We do not know the exact reason for this inconsistency, but it may possibly be due to variation of the batches of primary CEF and the serum used. In the nonadherent conditions, no significant activation of MAP kinase by UR2 and its mutants over the control cells was observed either, but the overall level of MAP kinase activity was reduced compared with that in adherent cells, consistent with the overall decreased tyrosine phosphorylation of cellular substrates in nonadherent cells. However, the MAP kinase activity in the mutant-expressing cells was comparable with that of the UR2-expressing cells in anchorage-independent conditions. The amount of MAP kinase protein immunoprecipitated and the amount of MBP used in the in vitro assay were similar in the control, UR2-expressing, and UR2 mutant-expressing cells in adherent and nonadherent conditions. The level of activation of MAP kinase was independently assessed by using a phosphospecific antibody recognizing only the phosphorylated and activated MAP kinase. The result confirmed the above observation in in vitro assay (data not shown). In a separate study, we investigated the effect of the MAP kinase inhibitor PD98059 on soft agar colony formation of UR2- and UR2 mutant-expressing cells (63). PD98059 had negligible effect on the colony forming ability of UR2 and its mutants, although the drug effectively inhibited MAP kinase at the concentrations used (63). Together, these results suggest that the MAP kinase signaling pathway cannot account for the differences in anchorage-independent growth among UR2 and its mutants.

Tyrosine Phosphorylation of PLCγ—Several studies have shown that PLCγ is activated by RPTKS (64). Our previous results (12) suggest that tyrosine 564 of the UR2 PTK may directly serve as the PLCγ recognition site or be critical for UR2 to interact with and activate PLCγ and that although phosphorylation of PLCγ is not essential for UR2-induced cell transformation, it could enhance cell transformation and anchorage-independent growth by v-Ros (12). We therefore examined the tyrosine phosphorylation of PLCγ by UR2 and its mutant proteins in adherent and nonadherent conditions (Fig. 3B). In adherent cells, the mutant PTKs (especially the F419 mutant) displayed reduced ability to phosphorylate PLCγ, compared with the UR2 protein. In nonadherent cells, such activity of UR2 was greatly diminished and became indistinguishable.
from either of the mutants, although all three viruses-infected CEF contained a higher level of phosphorylated PLCγ compared with that of the control cells. Thus, the status of the activation of PLCγ signaling cannot account for the impaired ability of the mutants to promote anchorage-independent growth either.

**Tyrosine Phosphorylation of Cytoskeletal Proteins**—Signaling initiated from the cell-cell and cell-matrix interactions could play an important role in the transforming ability and anchorage-independent growth of cells. The catenins, which connect the transmembrane cadherins to the actin cytoskeleton, are among the RPTK targets and are phosphorylated in response to stimulation by various growth factors (65). The tyrosine phosphorylation of β-catenin in v-src-transformed cells has been shown to result in reduction in cell-cell adhesion (66). We have previously shown that in monolayer F419 and DI are less effective in promoting tyrosine phosphorylation and interaction between various proteins involved in the formation of focal adhesion plaques and cell-cell interactions (12). To assess the role of cytoskeletal signaling in UR2-induced anchorage-independent growth, the phosphorylation of various molecules involved in cell-cell and cell-matrix interactions in adherent and nonadherent conditions was analyzed. The ability of UR2 and the mutants to promote tyrosine phosphorylation of β- and γ-catenin in adherent and nonadherent CEF was compared (Fig. 4, A and B). F419 and DI, particularly the former, were defective in their ability to induce phosphorylation of β- and γ-catenin in adherent conditions, confirming the previous findings (12), and the degree of phosphorylation was proportionally further reduced in the nonadherent conditions. However, the fold decrease of tyrosine phosphorylation of β-catenin in UR2-expressing cells was more pronounced than that in the mutant-expressing cells, when they were shifted from adherent to nonadherent conditions. Thus, whereas β-catenin could play a role in regulating anchorage-independent growth, it is not likely to be the predominant factor responsible for the differential ability of UR2, compared with the mutants, to promote anchorage-independent growth. These results suggest that attachment of cells to the substratum could affect the accessibility of these molecules for phosphorylation by v-Ros.

The interaction of integrins with the ECM at focal adhesions results in the tyrosine phosphorylation and activation of FAK, the recruitment of Src to FAK, and the subsequent phospho-

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**Fig. 2.** PTK activities of UR2 and its mutant Ros proteins in adherent and nonadherent CEF. A and B, 200 μg of cell lysates were immunoprecipitated (IP) with anti-Ros antibody and subjected to Western blotting with anti-phosphotyrosine (Anti-P-Tyr) Ab to determine intracellular tyrosine phosphorylation (A) or subjected to in vitro kinase assay to examine autophosphorylation (B) (top panels). In both cases, the blots were subsequently stripped and immunoblotted (IB) with anti-Ros to measure the protein amount (bottom panels). C, cellular substrate phosphorylation by v-Ros. 20 μg of the indicated total cell lysates were separated by SDS-PAGE, followed by Western blotting with anti-phosphotyrosine Ab (Anti-P-Tyr). D, cellular substrate phosphorylation by v-Src and v-Yes. 20 μg of the indicated total cell lysates were separated by SDS-PAGE, followed by Western blotting with anti-phosphotyrosine Ab.

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**Fig. 3.** Activity of MAP kinase and tyrosine phosphorylation of PLCγ in UR2- and UR2 mutant-expressing CEF grown in adherent and nonadherent conditions. A, 500 μg of cell lysates were immunoprecipitated (IP) with anti-Erk2 Ab. The immunoprecipitates were divided into two equal parts and subjected to in vitro MAP kinase assay using MBP as an exogenous substrate (top panel) or Western blotting (IB) with anti-Erk2 for detecting the protein amount (bottom panel). B, 500 μg of the indicated total cell lysates were separated by SDS-PAGE, followed by Western blotting with anti-phosphotyrosine Ab. C, 500 μg of the indicated total cell lysates were subjected to Western blotting with anti-phosphotyrosine Ab.

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Figure 4: A, 500 μg of cell lysates were immunoprecipitated (IP) with anti-PLCγ Ab and subjected to Western blotting (IB) with anti-PLCγ Ab to measure the PLCγ protein amount (bottom panel).
Tyrosine phosphorylation of proteins involved in cell-cell and cell-matrix signaling in UR2- and UR2 mutant-expressing CEF grown in adherent and nonadherent conditions. A and B, cell-cell signaling. 500 μg of cell lysates were immunoprecipitated (IP) with anti-β-catenin Ab (A) or anti-γ-catenin Ab (B) and subjected to Western blotting (IB) with anti-phosphotyrosine (Anti-P-Tyr) Ab (top panels). The blots were subsequently stripped and reprobed with the respective antibodies to check the protein amount (bottom panels). C-F, cell-matrix signaling. 500 μg of cell lysates were immunoprecipitated with anti-FAK (C), anti-paxillin (D), anti-p130Cas (E), or anti-Crk Ab (F) and subjected to Western blotting with anti-phosphotyrosine (Anti-P-Tyr) Ab (top panels). The blots were subsequently stripped and reprobed with the respective antibodies to check the protein amount (bottom panels).

Tyrosine Phosphorylation of Stat3—We have previously shown that activation of Stat3 is required for oncogenic Ros and insulin-like growth factor-1 receptor-induced cell transformation (44). We examined the tyrosine phosphorylation status of Stat3 in UR2-expressing and UR2 mutant-expressing CEF in adherent and nonadherent conditions (Fig. 6). The tyrosine phosphorylation level of Stat3 was significantly lower in F419-infected compared with DL- and UR2-infected cells in adherent conditions. However, this activity in DL-expressing cells was differentially reduced in nonadherent conditions compared with the UR2-expressing cells, although the latter also had a lower level of Stat3 phosphorylation compared with the counterpart adherent cells. These results show that anchorage to the ECM plays an important role for the increased IRS-1-associated PI 3-kinase activity in anchorage-dependent but not in anchorage-independent conditions. These results suggest that anchorage to the ECM is the major reason for the enhanced ability of UR2, compared with its mutants, to promote anchorage-independent growth. However, these attenuated signaling functions are likely to collectively impose restrictions on cell cycle progression of cells grown under nonadherent conditions.

IRS-1-associated PI 3-Kinase Activity—IRS-1 is the major effector of several RPTKs, including insulin and insulin-like growth factor-1 receptors, and its tyrosine phosphorylation is known to lead to activation of PI 3-kinase (69). We have previously shown that IRS-1 and PI 3-kinase are phosphorylated and activated by v-Ros (70). F419, but not DI, is impaired in inducing tyrosine phosphorylation of IRS-1 and IRS-1-associated PI 3-kinase activation in monolayer CEF (12). We compared the IRS-1-associated PI 3-kinase activity in UR2- and mutant-expressing cells in adherent and nonadherent conditions (Fig. 5). The results in adherent conditions confirmed our previous observation (12). However, in nonadherent conditions, both F419 and DI were impaired in inducing IRS-1-associated PI 3-kinase activity. UR2 was still able to significantly activate IRS-1-associated PI 3-kinase over that of the control cells, although at a reduced level compared with that in adherent conditions. Thus, DI was able to promote IRS-1-associated PI 3-kinase activity in anchorage-dependent but not in anchorage-independent conditions. These results suggest that anchorage to the ECM plays an important role for the increase of IRS-1-associated PI 3-kinase activity by v-Ros. Moreover, PI 3-kinase signaling defines a pathway that is activated in UR2- and impaired in the mutant-expressing cells in nonadherent conditions, suggesting that it may account, at least in part, for the loss of colony forming ability of the mutants.

Tyrosine phosphorylation of IRS-1 and PI 3-kinase were measured in monolayer CEF (Fig. 4). IRS-1 was phosphorylated in adherent and nonadherent conditions. IRS-1 phosphorylation was reduced in F419- and DI-expressing cells in nonadherent conditions and suggest that this impairment may contribute to the reduced ability of the mutants to mediate cell transformation and promote anchorage-independent growth.
Effect of dn Mutants of PI 3-Kinase and Stat3 on UR2-mediated Anchorage-independent Growth—Since UR2, but not the mutants, was able to significantly stimulate the IRS-1-associated PI 3-kinase activity and tyrosine phosphorylation of Stat3 in nonadherent conditions, we further assessed the requirement of these signaling pathways in UR2-mediated anchorage-independent growth. UR2-expressing cells were cotransfected with either control vector or the plasmid expressing dn p85, dn Stat3, or both, together with Us9-GFP, in a 10:1 molar ratio in favor of the former. Forty-eight hours after transfection, the GFP-expressing cells were isolated by cell sorting in the presence of 10 μg/ml concentration of antibiotics. Since the transfection efficiency obtained for UR2-transformed CEF was relatively low, such cell sorting allowed for enrichment of the cell population expressing the dn mutant molecules. Following sorting, the cells were allowed to recover by replating them and supplying them with fresh growth medium. To ensure that the dn constructs did not cause an increase in apoptotic cell death, we performed two independent experiments to measure the growth rates of the transfected cells. Each transfected cell population was counted and divided equally into two 35-mm dishes. One set, comprising UR2-expressing cells transfected with vector control, dn p85, or dn Stat3, was trypsinized and counted the following day. The second set was trypsinized and counted 3 days later, and the fold growth was determined. The fold growth of the UR2-expressing cells transfected with vector control, dn p85, or dn Stat3, was trypsinized and counted the following day. The second set was trypsinized and counted 3 days later, and the fold growth was determined. The fold growth of the UR2-expressing cells transfected with vector control, dn p85, or dn Stat3, was trypsinized and counted the following day. The second set was trypsinized and counted 3 days later, and the fold growth was determined. The fold growth of the UR2-expressing cells transfected with vector control, dn p85, or dn Stat3, was trypsinized and counted the following day. The second set was trypsinized and counted 3 days later, and the fold growth was determined.
dn mutants was scored at an early stage (7 days) for UR2-induced colony formation considering the possibility that the cells might lose expression of the dominant negative mutants due to loss of the transgene. Both mutants significantly decreased the anchorage-independent growth of UR2-expressing cells. Whereas dn p85 reduced the colony forming ability of UR2 to about 59% and dn Stat3 reduced the same to about 32%, together they reduced the UR2-mediated colony formation to about 17% (Fig. 7A). Parallel cultures of the sorted cells were amplified and used for analyzing protein expression of the dn molecules (Fig. 7C). These results show that inhibition of PI 3-kinase and Stat3 signaling greatly reduced the UR2-induced anchorage-independent growth. The role of PI 3-kinase in UR2-induced cell transformation was independently confirmed in a separate study, where we showed that pharmacological inhibitors of PI 3-kinase significantly inhibited UR2-induced colony formation (63). Taken together, these results confirm the essential role of the PI 3-kinase and Stat3 signaling pathways in UR2-mediated anchorage-independent growth.

**Effect of ca Mutants of PI 3-Kinase and Stat3 on the Colony Forming Ability of F419 and DI**—Next, we investigated whether constitutively activated mutants of PI 3-kinase and Stat3, alone or in combination, could rescue the anchorage-independent growth of F419- and DI-expressing CEF. F419- and DI-expressing cells were co-transfected with either control vector or the plasmid expressing ca PI 3-kinase (BD110), ca Stat3 (Stat3-C), or both, together with Us9-GFP in a 10:1 molar ratio as described above. To evaluate the basal level of colony forming ability of the ca molecules, control CEF were transfected with the plasmid expressing BD110 or Stat3-C or with both plasmids, together with Us9-GFP. Forty-eight hours after transfection, the GFP-expressing cells were isolated by cell sorting as described above. Following recovery in monolayer culture conditions, the cells were subjected to soft agar colony assays. Control CEF transfected with plasmids expressing BD110, Stat3-C, or both did not show any colony formation under our conditions (data not shown). The colony formation by F419-expressing cells transfected with BD110 or Stat3-C plasmids alone was enhanced about 5-fold compared with transfection with the control vector (Fig. 7B). F419-expressing cells transfected with both BD110 and Stat3-C showed a 9.2-fold enhancement in colony formation compared with the control vector-transfected cells. DI-expressing cells transfected with BD110 or Stat3-C showed about 2- or 3-fold increase, respectively, over the control (Fig. 7B). DI-expressing cells transfected with both BD110 and Stat3-C showed about 4-fold enhancement in colony formation compared with the control vector-transfected cells. The rescuing effect of Stat3-C and BD110 appeared to be additive. The expression levels of the ca molecules in the transfections are shown in Fig. 7C. These results showed that activation of the PI 3-kinase and Stat3 signaling pathways, that could not be activated in F419- and DI-expressing cells in nonadherent conditions, rescued the anchorage-independent growth of these cells. The differential activation of these pathways by UR2 in CEF deprived of ECM adhesion could therefore account for its colony forming ability.

**Analysis of G1 Phase Cyclin-associated Cdk Activities**—Since UR2-expressing cells displayed faster cell cycle progression than the control and F419 and DI mutant-expressing CEF in nonadherent conditions, we analyzed the activation status of the Cdns in these cells. We compared the relative Cdk activities in UR2- and UR2 mutant-expressing CEF in adherent and nonadherent conditions. The cyclin D1-associated Cdk4/6 kinase activity was measured by using a truncated Rb protein as an exogenous substrate (Fig. 8A). In adherent conditions, UR2 induced the increase of such activity compared with that of the control CEF. The mutants also induced increased cyclin D1-associated Cdk4/6 activity, although they were not as potent as UR2. In the absence of ECM-mediated signaling in nonadherent conditions, no significant cyclin D1-associated Cdk4/6 activity could be detected in the control as well as in the UR2- and mutant-expressing CEF. Cyclin E- and cyclin A-associated
Cdk2 activities were measured using histone H1 protein as the exogenous substrate (Fig. 8, B and C). The cyclin E-associated Cdk2 activity was also considerably attenuated in the nonadherent cells compared with that of the adherent cells, but it was indistinguishable among control and UR2- and UR2 mutant-expressing CEF in either culture condition. Thus, alteration of neither cyclin D1-associated Cdk4/6 nor cyclin E-associated Cdk2 activities could account for the UR2-mediated promotion of cell cycle progression in nonadherent conditions. Cyclin A-associated Cdk2 activity was enhanced in all three virus-infected CEF compared with the control cells in adherent conditions, although the mutants, especially F419, were attenuated in promoting such activity (Fig. 8C). Similar to the patterns of activation of PI 3-kinase and Stat3, F419 and DI were differentially impaired in nonadherent conditions in their ability to induce cyclin A-associated Cdk2 activity. Although significantly diminished, UR2 retained a considerable degree of such activity. Thus, the accelerated cell cycle progression in nonadherent UR2-expressing cells appears to correlate with the increased cyclin A-associated Cdk2 activity in these cells. The expression levels of the Cdkks, Cdk2, Cdk4, and Cdk6 and of the cyclins A and E were comparable in UR2- and UR2 mutant-expressing cells in adherent and nonadherent conditions (data not shown). We did not see any significant enhancement of the Rb phosphorylation either in UR2-expressing cells compared with the control or mutant-expressing cells in nonadherent conditions, consistent with the comparable cyclin D1-associated Cdk4/6 kinase activity in those cells (data not shown). Since the expression of cyclin A appears to be ECM-independent, it is likely that the cyclin A-associated Cdk2 activity is regulated by alterations in the levels of the cyclin-dependent kinase inhibitors p21 and/or p27. However, we were unable to detect the endogenous levels of p21 and p27 in our experimental conditions (data not shown).

**Effect of the Inhibition of PI 3-Kinase or Stat3 Signaling on UR2-induced Cyclin A-associated Cdk2 Kinase Activity**—Next, we investigated whether enhanced PI 3-kinase or Stat3 signaling in nonadherent UR2-expressing cells contributed to the enhanced cyclin A-associated Cdk2 kinase activity in these cells. UR2-expressing cells were co-transfected with a control vector or the plasmid expressing dn Stat3 together with Us9-GFP in a 10:1 molar ratio, and the GFP-expressing cells were isolated by cell sorting as described above. After initial recovery, the sorted cells were grown in MC medium for 48 h, and their cyclin A-associated Cdk2 kinase activities were analyzed. Transfection of dn Stat3 did not lead to altered cyclin A-associated Cdk2 kinase activity in the nonadherent UR2-infected cells, although the expression level of dn Stat3 was sufficient to inhibit UR2-mediated anchorage-independent growth in a parallel experiment (data not shown). By contrast, treatment of nonadherent UR2-expressing cells with the PI 3-kinase inhibitor LY294002 resulted in a decrease in cyclin A-associated Cdk2 kinase activity to a level comparable with that of the nonadherent F419- and DI-expressing cells (Fig. 8D). The colony forming ability of UR2-expressing CEF was reduced to 50% and 35% of the control in the presence of 5 and 10 μM LY294002, respectively (63). Thus, the induction of cyclin A-associated Cdk2 kinase activity in UR2-expressing cells in an-

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**Fig. 8.** Cdk activities in UR2- and UR2 mutant-expressing CEF grown in adherent and nonadherent conditions and effect of LY294002 on UR2-induced cyclin A-associated Cdk2 activity. A, 500 μg of cell lysates were immunoprecipitated (IP) with anti-cyclin D1 Ab and subjected to in vitro Cdk2 kinase assay using a retinoblastoma protein as an exogenous substrate (top panel) followed by Western blotting (IB) with anti-Rb to check the Rb protein amount (bottom panel). B, 500 μg of cell lysates were immunoprecipitated with anti-cyclin E Ab and subjected to in vitro Cdk2 kinase assay using histone H1 as an exogenous substrate (top panel). The bottom panel represents Coomassie Blue staining of the gel to monitor the amount of histone H1 protein. C, 500 μg of cell lysates were immunoprecipitated with anti-cyclin A Ab and subjected to an in vitro Cdk2 kinase assay using histone H1 as an exogenous substrate (top panel). The bottom panel represents Coomassie Blue staining of the gel to monitor the amount of histone H1 protein.
chorage-independent conditions requires the activation of IRS-1-associated PI 3-kinase activity but not the activation of Stat3, and the increase of Cdk2 activity could play an important role in the anchorage-independent growth of these cells.

Role of Increased Cyclin A-associated Cdk2 Activity in An-
chorage-independent Growth

To directly assess the contribution of the increased cyclin A-associated Cdk2 activity in anchorage-independent growth, the UR2-expressing cells were treated with different doses of the Cdk2 inhibitor roscovitine. At a concentration of 50 \( \mu M \) roscovitine significantly inhibited UR2-mediated anchorage-independent growth (Fig. 9A). The cyclin A-associated Cdk2 kinase activity in the nonadherent UR2-expressing cells at this concentration of roscovitine was reduced to a level comparable with that in control CEF (Fig. 9B). Cyclin E-associated Cdk2 activity in UR2-expressing cells was not significantly affected up to 60 \( \mu M \) of roscovitine (data not shown). At a concentration of 60 \( \mu M \), roscovitine abolished UR2-mediated anchorage-independent growth and grossly inhibited the cyclin A-associated Cdk2 kinase activity in nonadherent UR2-expressing cells. We confirmed these results using another Cdk2 inhibitor, olomoucine. Olomoucine significantly inhibited UR2-mediated anchorage-independent growth (Fig. 9A) as well as the cyclin A-associated Cdk2 activity in nonadherent UR2-expressing cells (Fig. 9B) at a concentration of 60 \( \mu M \).

Taken together, the differential impairment in the induction of such Cdk2 kinase activity in the F419- and DI-expressing cells as a consequence of their failure to activate PI 3-kinase signaling in these cells is likely to account for the delayed cell cycle progression and reduced anchorage-independent growth of these cells. On the other hand, the induction of cyclin A-associated Cdk2 kinase activity in UR2-expressing cells in nonadherent conditions appears to account for the enhanced cell cycle progression of these cells, thus promoting their anchorage-independent growth.

DISCUSSION

Although anchorage-independent growth is the best in vitro correlate of tumorigenicity (13), most studies on the activation of signaling pathways in oncogene-induced cell transformation have used cells grown in adherent conditions in monolayer cultures. In this study, we employ the methylcellulose system that enables the growth of transformed cells in nonadherent conditions and the subsequent recovery of the cells for analysis of the signaling pathways with respect to their activation status and requirement for anchorage-independent growth. We have compared the signaling pathways activated by UR2 and its mutants, F419 and DI, in adherent and nonadherent conditions in an attempt to identify those pathways that are important for UR2-induced anchorage-independent growth. We have previously shown that F419 and DI retain wild type protein-tyrosine kinase and mitogenic activities in monolayer cultures but have dramatically reduced ability to promote colony formation in soft agar (12). These mutants serve as invaluable tools allowing one to delineate signaling pathways, leading to mitogenicity in monolayer versus anchorage-independent growth. In monolayer cultures, both F419 and DI mutants were found to be impaired in their ability to phosphorylate a series of...
cytoskeletal and cell-cell interacting proteins, and F419 was additionally impaired in activating the PI 3-kinase pathway (12). Our results in methylcellulose-containing medium show that tyrosine phosphorylation levels of all signaling molecules, particularly the components of ECM integrin-mediated signaling, are globally reduced in control and in UR2- and UR2 mutant-infected CEF in nonadherent conditions despite the comparable levels of Ros expression and PTK activity in either growth condition. This phenomenon was also observed with the nonreceptor PTK oncogenic v-Src and v-Yes. These results indicate that ECM- and integrin-mediated signaling is important in general for substrate phosphorylation, even by constitutively activated PTKs. Among the major signaling molecules, IRS-1-associated PI 3-kinase activity and Stat3 phosphorylation are differentially impaired in the mutant-expressing cells compared with the UR2-expressing cells in nonadherent conditions. Thus, the nonadherent growth of UR2-expressing cells may be a consequence of the ability of UR2 to induce activation of specific signaling pathways such as PI 3-kinase and Stat3.

Our results using dominant negative and constitutively active PI 3-kinase and Stat3 strongly suggest that these pathways are indeed important for Ros-induced anchorage-independent growth and cell transformation. The activation of these pathways is apparently able to overcome the restrictions on cell cycle progression imposed by global attenuation of signaling functions in the absence of ECM-integrin engagement.

Our data show that UR2-expressing cells are able to promote anchorage-independent growth due to faster cell cycle progression instead of decreased rate of cell death as compared with control and the mutant-expressing CEF. This is consistent with the idea that in suspension fibroblasts usually undergo cell cycle arrest (71–73), whereas epithelial and endothelial cells undergo anoikis (23, 24) and that oncogenic transformation of these cells can result in increased cell proliferation (47, 74) or resistance to apoptosis (23, 37), respectively, in the absence of ECM attachment.

Integrins transduce many signals that impinge upon growth regulatory pathways (18, 19, 20). These include activation of tyrosine kinases such as Fak, Src, and c-Abl; serine/threonine kinases such as MAP kinase, c-Jun N-terminal kinase, and protein kinase C; PI 3-kinase; small GTPases; and intracellular ions such as protons (pH) and calcium. There is a considerable overlap between RPTKs and integrins with respect to the recruitment of downstream signaling substrates that lead to the activation of these signaling pathways. The integrin-mediated signaling thus functions to synergize with and augment the growth factor receptor-mediated signaling. The greatly decreased tyrosine phosphorylation of cellular substrates in anchorage-independent conditions may be due to the absence of integrin engagement with the ECM and subsequent lack of recruitment of these substrates to focal adhesions in these cells. This may result in reduced availability of the substrates in the vicinity of RPTKs at the plasma membrane.

Constitutive MAP kinase activation has been shown in certain cell systems to be important for the establishment and/or maintenance of transformation and tumorigenesis (75–77). The ability of human breast cancer MCF-7 cells to respond to insulin-like growth factor-1 and initiate DNA synthesis in the absence of substratum attachment has been partially attributed to activation of the MAP kinase pathway (78). Activation or overexpression of PLCγ has been implicated in stimulating DNA synthesis and promoting cell transformation by epidermal growth factor and platelet-derived growth factor receptors (79, 80). Our results indicate that the activation of MAP kinase and the tyrosine phosphorylation of PLCγ are not sufficient for the differential ability of UR2 to promote anchorage-independent growth of CEF, since the mutants F419 and DI are not impaired in their ability, compared with UR2, to activate these pathways under nonadherent conditions. This is consistent with the observation that the MAP kinase inhibitor PD98059 has little effect on UR2-induced colony formation (63).

Cytoskeletal signaling mediates important cellular functions that may contribute to cellular transformation. A compensatory role of cadherin-mediated intercellular adhesions and signaling in anchorage-independent growth has been demonstrated in human squamous carcinoma HSC-3 cells (81). These cells cease proliferation and enter the apoptotic pathway when suspended as single cells but survive and proliferate when permitted to form E-cadherin-mediated multicellular aggregates in suspension. FAK has been found to suppress apoptosis caused by the deprivation of anchorage in transformed cells (37). Adenoviral gene transduction of the C-terminal domain of FAK, which down-regulates FAK function, caused apoptosis in breast cancer cells that are otherwise viable in the absence of ECM attachment. We have previously shown that in adherent CEF, the F419 and DI proteins are less effective than UR2 in promoting tyrosine phosphorylation and interaction among various proteins involved in the formation of cell-cell interactions and focal adhesions plaque (12), suggesting that signaling involving such cytoskeletal proteins may play an important role in UR2-mediated cell transformation, especially in anchorage-independent growth. Although tyrosine phosphorylation levels of the catenins and focal adhesion proteins are decreased in nonadherent conditions, especially for the latter, the differences between UR2 and its mutants are maintained proportionally in nonadherent conditions compared with the differences in adherent conditions, suggesting that while these molecules could be contributing to growth, they are not likely the major factors for differentiating the ability of UR2 from its mutants in promoting anchorage-independent growth.

In this study, the IRS-1-associated PI 3-kinase activity and Stat3 tyrosine phosphorylation define signaling pathways that are activated in UR2-expressing cells and differentially impaired in the mutant-expressing cells in anchorage-independent conditions and may account mainly for the loss of colony forming ability of the mutants. An analysis of whether activation of these pathways and their downstream signaling molecules is sufficient for transformation by UR2 would therefore provide useful clues about the molecular mechanisms underlying anchorage-independent growth. For this purpose, we have tested the ability of dominant negative mutants of PI 3-kinase and Stat3 to inhibit UR2-mediated cell transformation as well as the ability of their constitutively activated mutants to rescue colony formation by F419 and DI. Dominant negative mutants of PI 3-kinase and Stat3 when introduced together are more potent in reducing UR2-mediated colony formation than when introduced individually. Likewise, constitutively active mutants of PI 3-kinase and Stat3 are able to rescue F419- and DI-mediated colony forming ability when transfected individually and do so synergistically when transfected together. Our results confirm the essential role of PI 3-kinase and Stat3 signaling in Ros-induced anchorage-independent growth and indicate that the two pathways synergize to promote colony formation mediated by UR2. We have shown that in NIH3T3 cells expressing ER2, an epidermal growth factor receptor-Ros chimeric receptor, overexpression of c Stat3 conferred partial resistance to the inhibition of Ros-induced anchorage-independent growth by LY294002, suggesting a partial overlap of the functions between PI 3-kinase and Stat3 signaling in those cells (63). Our current finding of the synergistic effect of PI 3-kinase and Stat3 signaling pathways suggests that their functions are not completely overlapping.
We have previously shown that PI 3-kinase and Stat3 are important for anchorage-independent growth of NIH 3T3 cells in soft agar induced by a chimeric epidermal growth factor receptor-ROS RPTK in response to epidermal growth factor (44, 63). Thus, activation of PI 3-kinase and Stat3 in nonadherent UR2-expressing cells may compensate for the lack of ECM- and integrin-mediated signaling and be able to lead to up-regulation of the proteins involved in cell cycle progression. Our current results show that whereas cyclin D1-associated Cdk4/6 and cyclin E-associated Cdk2 activities and the phosphorylation of Rb are similar in UR2- and its mutant-expressing cells in nonadherent conditions, F419 and DI are differentially impaired in their ability to induce cyclin A-associated Cdk2 activity. Cyclin A, the last cyclin to be activated in the G1 phase, controls normal progression into the S phase. Several studies have demonstrated an essential role for cyclin A in anchorage-independent growth. Rh-dependent down-regulation of cyclin A has been shown to be involved in the suppression of anchorage-independent growth by the dros tumor suppressor gene, whose mRNA expression in turn is down-regulated by v-Src and v-K-Ras (82). The ability of bovine papillomavirus type 4 (BPV-4) E8 protein to promote the anchorage-independent growth of NIH3T3 cells has been measured in cells maintained in short-term suspension culture and low serum and is associated with up-regulation of cyclin A expression, cyclin A-associated kinase activity, and deregulated expression of the CK1 p27 (83). The expression of constitutively active Cdc42 or Ras in primary mouse embryo fibroblasts, resulting in increased cyclin A gene transcription, is sufficient for anchorage-independent cell cycle progression of these cells (84). The fibroblast line ER1-2 is specifically mutated in Ras oncogene-mediated anchorage-independent growth (85). Although Ras can override the adhesion requirements for RB phosphorylation and cyclin E-dependent kinase activity in those cells in the absence of ECM adhesion, the cells remain adhesion-dependent for cyclin A expression, and anchorage-independent growth of those cells can be rescued by ectopic expression of cyclin A (47). Although the expression level of cyclin A was not affected in CEF in nonadherent conditions, our data show that inhibition of cyclin A-associated Cdk2 kinase activity results in decreased UR2-mediated anchorage-independent growth of CEF. These observations suggest that in this case the Cdk2 activity is likely to be regulated by the Cdk inhibitors.

Several studies have demonstrated a role for PI 3-kinase signaling in the control of cell cycle progression. Our studies show that inhibition of PI 3-kinase by LY294002 in nonadherent UR2-expressing cells leads to reduced cyclin A-associated Cdk2 kinase activity in these cells. Inhibition of PI 3-kinase by LY294002 has been shown to induce a senescence-like growth arrest mediated by the increase of p27 levels and inhibition of Cdk2 in mouse primary embryo fibroblasts (86). The lipid phosphatase PTEN can suppress the tumorigenicity of human glioblastoma U87MG cells by blocking cell cycle progression in the G2 phase via up-regulation of p27 (87). The phosphorylation of the Cdk inhibitor p21 by Akt, the downstream effector of PI 3-kinase, has been shown to result in the cytoplasmic localization of p21 in HER-2/neu-overexpressing cells, thereby promoting cell growth (88). This may account for our observation of the PI 3-kinase-dependent increase of cyclin A-associated Cdk2 kinase activity in the UR2-transformed cells. Activation of PI 3-kinase has been shown to be sufficient for cell cycle entry but not for progression through the entire cell cycle (33). Thus, it is likely that PI 3-kinase may synergize with other signaling pathways, such as Stat3, to enhance the cell cycle progression.

In summary, we have characterized the signaling pathways contributing to v-Ros-mediated cell transformation using transformed cells grown independent of anchorage to the extracellular matrix. We show that the activation of PI 3-kinase and Stat3 signaling functions is essential for UR2-induced anchorage-independent growth. Furthermore, we show that UR2 promotes anchorage-independent growth by accelerating the cell cycle progression of CEF, which in part could be accounted for by the PI 3-kinase-dependent increase of cyclin A-associated Cdk2 activity.

Overall, we believe that deprivation of ECM anchorage globally reduces a multitude of signaling pathways such that they are below the threshold of being able to sustain the cell cycle progression of normal fibroblasts despite the presence of abundant growth factors. Oncogenic PTKs, such as Ras, are able to continue to activate certain critical signaling pathways such as PI 3-kinase and Stat3, thus overcoming the stress and allowing cell cycle progression under suboptimal conditions, thereby promoting anchorage-independent growth.

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Matrix-independent Activation of Phosphatidylinositol 3-Kinase, Stat3, and Cyclin A-associated Cdk2 Is Essential for Anchorage-independent Growth of v-Ros-transformed Chicken Embryo Fibroblasts
Shailaja Uttamsingh, Cong S. Zong and Lu-Hai Wang

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