Multiple Ras Effector Pathways Contribute to G1 Cell Cycle Progression*

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The involvement of Ras in the activation of multiple early signaling pathways is well understood, but it is less clear how the various Ras effectors interact with the cell cycle machinery to cause G1 progression. Ras-mediated activation of extracellular-regulated kinase/mitogen-activated protein kinase has been implicated in cyclin D1 up-regulation, but there is little extracellular-regulated kinase activity during the later stages of G1, when cyclin D1 expression becomes maximal, implying that other effector pathways may also be important in cyclin D1 induction. We have addressed the involvement of Ras effectors from the phosphatidylinositol (PI) 3-kinase and Raf-Ral-GDS families in G1 progression and compared it to that of the Raf/mitogen-activated protein kinase pathway. PI 3-kinase activity is required for the expression of endogenous cyclin D1 and for S phase entry following serum stimulation of quiescent NIH 3T3 fibroblasts. Activated PI 3-kinase induces cyclin D1 transcription and E2F activity, at least in part mediated by the serine/threonine kinase Akt/PKB, and to a lesser extent the Rho family GTPase Rac. In addition, both activated Ral-GDS-like factor and Raf stimulate cyclin D1 transcription and E2F activity and act in synergy with PI 3-kinase. Therefore, multiple cooperating pathways mediate the effects of Ras on progression through the cell cycle.

Upon reentry of cells into the cell cycle and throughout the G1 phase, mitogenic signals are integrated through the GTPase Ras. Inhibition of Ras function by microinjection of Ras-neutralizing antibodies or by inducible expression of dominant negative Ras arrests cycling cells in G1 and prevents growth factor stimulated cells from leaving G0 to reenter the cell cycle (1–4). The requirement of Ras function during the G0/G1 transition seems to be conserved from yeast, in which it is necessary for spore germination (5). Microinjection of anti-Ras antibodies has demonstrated a requirement for Ras up to 2 h prior to S phase entry (6, 7).

Overexpression of mutationally activated Ras leads to cellular transformation and a shortening of the G1 phase of the cell cycle (8). However, transformation of cells by activated Ras requires other genetic changes, as only immortal cells that have lost cell cycle checkpoints, such as those imposed by cyclin-dependent kinase (cdk)1 inhibitors, retinoblastoma protein, and p53, can be transformed by Ras alone. The expression of activated Ras in primary cells leads to cell cycle arrest via up-regulation of cdk inhibitors and p53. The resulting phenotype resembles that of cellular senescence (9). In mouse embryo fibroblasts (10, 11) and rat Schwann cells (12), the Ras effector Raf appears to be sufficient to mediate this effect via induction of p21Waf/Cip1. Thus, Ras exerts both positive and negative effects on cell growth, depending on cellular context.

Recent work from this and other laboratories has shown that Ras is able to interact with multiple effector enzymes, including the Raf protein kinases, the Ral-GDS family of guanine nucleotide exchange factors for Ral, and type I phosphoinositide 3-kinases (13). The functions of multiple Ras effectors are required for cellular transformation; in addition to Raf, both the PI 3-kinase and the Ral-GDS pathways cooperate to achieve efficient transformation of immortalized cells (14, 15). Therefore, several Ras effector pathways may interface with the cell cycle machinery.

Ras has been implicated in the positive regulation of the cyclin D1 promoter. The conditional expression of oncogenic Ras induces cyclin D1 protein production in growth factor-deprived cells (16). However, the resulting cyclin D1/cdk4 complexes may remain inactive in the absence of growth factors (17). Activation of Raf-ER, a conditionally active fusion of Raf to the hormone binding domain of the estrogen receptor, and conditional expression of an activated form of the MAP/ERK kinase, MEK, reproduce the effect of oncogenic Ras on cyclin D1, and under certain conditions this signal can be blocked by co-expression of a MAP kinase phosphatase or dominant-interfering mutants of ERK. Therefore, these Ras effects have been attributed to the sequential activation of Raf kinase, MEK, and ERK (18–20). However, there are circumstances under which ERK activity may be dispensable for cyclin D1 production as the MEK inhibitor PD 98059 does not inhibit serum-induced cyclin D1 expression in IIC9 fibroblasts (21).

It has been suggested that low levels of Raf kinase activity promote proliferation by inducing cyclin D1 and strong Raf activity inhibits cell cycle progression through production of cdk inhibitors (9–12). However, it is also possible that the divergent effects of Ras on proliferation are mediated by distinct effector proteins, which could be subject to independent regulation by cross-talk with other signaling pathways. Therefore, we investigated which of the known Ras effectors would interface with the G1 cell cycle machinery. We find that activation of PI 3-kinase is required for cyclin D1 protein expression and S phase entry in fibroblasts. The PI 3-kinase pathway

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1 The abbreviations used are: cdk, cyclin-dependent kinase; MAP, mitogen-activated protein; ERK, extracellular-regulated kinase; MEK, MAP/ERK kinase; PI, phosphatidylinositol; PIP3, PI 3,4,5-trisphosphate; Rb, retinoblastoma; Rif, Ral-GDS-like factor; Ral-GDS, Ral guanine nucleotide dissociation stimulator; PKB, protein kinase B; TOR, target of rapamycin.
also contributes to the activation of E2F-dependent transcription underlining its importance for progression through G1. An activated form of Raf-GDS-like factor (Rlf), which has recently been implicated in the transcriptional induction of the c-fos proto-oncogene, also strongly activated cyclin D1 transcription. Thus, multiple pathways in addition to Raf/ MAP kinase mediates the effects of Ras on cyclin D1 expression and E2F transcriptional activity in order to drive G1 progression.

MATERIALS AND METHODS

Plasmids—The reporter plasmid pE2CAT and has been described previously (31). The luciferase reporter pGL2-lucD1 contains 1.8 kilobase pairs of the human cyclin D1 promoter, including the TATA box inserted into the Smal site of pGL2 (Promega) and was provided by R. Assoian. Expression vectors for Raf-GDS-like factor (52) and Rabla (53) have been described. Rabla F39L was generated using the mutagenic oligonucleotide primer CATGTCAGTAGAGATGAGGACTATG on pMT3-Rabla using standard procedures.

The activated pSG5 gag-Akt/PKB (54) and dominant-negative Akt/ PKB (55) constructs were kindly provided by B. Burgering. Expression plasmids for activated PI 3-kinase (25), V12 Ras, V12 Rac, and V12 Cdc42 have been described (15). Ras CAAAX DD is an activated form of c-Raf-1 in which Raf is localized to the membrane by a farnesylation sequence from H-Ras and also by mutation of two tyrosine residues, 340 and 341, to aspartic acids; it was kindly provided by Raichard Marais and Chris Marshall.

Cell Culture and Transfections—NIH 3T3 cells were maintained in 10% calf serum and starved for 48 h in 0.15% calf serum where indicated. LY294002 was used at 20 μM unless indicated otherwise. The final concentration of PD 98059 (Biomol) was 30 μM, and rapamycin was used at 50 nM. Cells were preincubated with inhibitors for 10 min prior to addition of calf serum to 10%. LipofectAMINE was used (Santa Cruz). Anti-phospho-ERK was purchased from Promega. Ras was detected using a polyclonal serum (Pharmingen), and Rb was detected using a polyclonal serum and 1mM benzamidine. The suspension was aliquoted, snap-frozen, and stored at -80°C.

Cells were washed twice in phosphate-buffered saline and treated with 5% glycerol, 0.2 mM EDTA, and 0.1% Triton X-100. Bound Ras was quantified by Western blotting. The serine/threonine kinase Akt/PKB is activated by phosphorylation of Thr308 and Ser473 by PDK1. Assoian. Expression vectors for Ral-GDS-like factor (52) and Rabla (53) have been described. Rabla F39L was generated using the mutagenic oligonucleotide primer CATGTCAGTAGAGAGGTACTATG on pMT3-Rabla using standard procedures.

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RESULTS

Sustained Ras and Akt/PKB Activation during G1—Ras function has been shown to be required throughout G1 up to the restriction point, after which cells become committed to enter S phase. In order to determine Ras activity at later stages in G1, we made use of the high affinity interaction of the active GTPase with the Ras binding domain of Raf kinase (22). Quiescent NIH 3T3 cells were stimulated with serum, and GTPbound Ras was purified based on its ability to bind to recombinant fusion protein containing the Ras binding domain of Raf. Ras activation was found to be biphasic with a second peak at G1 peak of activity that was maximal at about 2–4 h after exit from quiescence (Fig. 1A). Whereas the first peak of Ras activity up to about 30 min after stimulation correlated with ERK phosphorylation, little ERK activity could be detected at 4 h (Fig. 1B) (see also Ref. 22). This is likely to be due to the induction of MAP kinase phosphatases as a result of the early phase of growth factor receptor and Ras activation (23).

One of the functions in cell cycle progression attributed to Ras in cells released from quiescence is the induction of cyclin D1 protein as a consequence of ERK activation. However, cyclin D1 expression became detectable only at 4 h after stimulation and became maximal at 6–8 h (see Fig. 3, A and B), consistent with previous reports (24). Thus, cyclin D1 induction correlates with the second phase of Ras activity, which is not associated with ERK activation.

The serine/threonine kinase Akt/PKB is activated by phosphorylation of Thr308 and Ser473 by PDK1, which is generated upon activation of the Ras effector PI 3-kinase. In order to determine whether the time course of Akt/PKB activation is consistent with a role in G1 progression, its kinase activity was determined at various times during the G1 phase. As shown in Fig. 1C, Akt/PKB activity was sustained during G1, and its period of activity encompassed the second peak of Ras activation.

PI 3-kinase Is Required for G1 Progression—In order to determine which Ras effector pathways are required for G1 progression, we made use of chemical inhibitors for MEK (PD 98059) and PI 3-kinase (LY 294002). Because it is known that LY 294002 also inhibits some relatives of PI 3-kinase, such as TOR, the specificTOR inhibitor rapamycin was also used.
Quiescent NIH 3T3 cells were preincubated with inhibitor and then stimulated with serum in the presence of inhibitor.

Fig. 2A shows the cell cycle distribution at 14 and 20 h after serum treatment. Whereas both PD 98059 and rapamycin caused delays in S phase entry, many cells treated with either inhibitor were able to initiate DNA synthesis by 14 h. However, in the presence of LY 294002, cells had not progressed into S phase at all after 14 h. At 20 h, more than 75% of the LY 294002-treated cells were still in G0/G1, compared with well under 50% for control cells and those treated with PD 98059 or rapamycin. In order to determine the effectiveness of the drug treatments, cell lysates were immunoblotted with anti-phospho-Akt or anti-phospho-ERK antibodies. Levels of the activity of these downstream effectors of PI 3-kinase and Raf were less than 5% of untreated serum controls for Akt in the presence of 20 \( \mu \text{M} \) LY294002 or 15% for ERK in the presence of 30 \( \mu \text{M} \) PD98059 at all time points (data not shown).

Fig. 2B shows the effect of the PI 3-kinase inhibitor on unsynchronized cells. The presence of LY 294002 for 48 h induced a level of arrest similar to that observed in growth factor deprived cells. The half-maximal inhibition of G1 progression was achieved at 4 \( \mu \text{M} \) LY 294002, which is similar to the IC50 for inhibition of insulin stimulated Akt/PKB activity (Fig. 2C)(25). These results suggest that PI 3-kinase activity is essential for the progression of cells through the G1 phase of the cell cycle.

**Fig. 2.** LY294002 prevents S phase entry. **A,** At 0, 14, and 20 h after serum stimulation of quiescent NIH 3T3 cells in the presence of the indicated inhibitors of PI 3-kinase (LY294002), MEK (PD98059), or TOR (rapamycin), cells were fixed and stained with propidium iodide, and their position in the cell cycle was determined by FACS analysis. **B,** LY294002 was added to unsynchronized cells in the presence of serum and the cell cycle distribution was determined after 24 h and 48 h. The distribution of untreated (cells in the presence of serum) and serum-deprived cells was analyzed in parallel. **C,** unsynchronized cells were grown for 24 h in serum in the presence of different concentrations of LY294002. The arrest observed after 48 h in the presence of 20 \( \mu \text{M} \) was plotted as 100%.

**Effect of PI 3-kinase and MEK Inhibition on Endogenous Cyclin D1 Expression**—One of the earliest effects of serum stimulation leading to cell cycle reentry in fibroblasts is the expression of cyclin D1 protein. In order to determine the effects of the various inhibitors on cyclin D1 expression, NIH 3T3 cells were treated with inhibitors as above, and the expression of cyclin D1 was analyzed. Fig. 3A shows that the presence of the PI 3-kinase inhibitor LY 294002 severely inhibited cyclin D1 expression at all time points studied. The presence of the MEK inhibitor PD 98059 led to a slightly reduced expression of cyclin D1 relative to uninhibited cells at 7 h, but there was no effect at later time points. The TOR inhibitor rapamycin caused some decrease in cyclin D1 expression at 7 and 14 h, but by 20 h, this effect was negligible. At all times, rapamycin was less inhibitory for cyclin D1 expression than was LY 294002, suggesting that the effects of the PI 3-kinase inhibitor were not connected with inhibition of the target of rapamycin. The PI 3-kinase inhibitor LY 294002 thus inhibits one of the earliest events in G1.

In order to determine the length of time in G1 during which PI 3-kinase activity is required for cyclin D1 expression, the inhibitor was added at different times after release of cells from...
stabilization by serum treatment. Fig. 3B shows that about 8 h after serum treatment, cyclin D1 expression becomes independent of PI 3-kinase activity but that inhibition of PI 3-kinase at any time during the first 6 h of serum treatment results in inhibition of cyclin D1 expression. Inhibition of MEK through the use of PD98059 has only a small effect on cyclin D1 expression, which is lost if inhibitor treatment is delayed until 4 h after serum addition, suggesting that the low levels of ERK activity in late G1 are not important for cyclin D1 expression.

Fig. 3C shows that the concentration of rapamycin used in this experiment was sufficient to inhibit phosphorylation of p70S6K, the downstream target of TOR activity. The presence of LY 294002 did not lead to detectable p70s6k inhibition, reflecting the fact that although PI 3-kinase contributes to the regulation of p70s6k, there are multiple pathways involved including some utilizing protein kinase C that are not sensitive to inhibition of PI 3-kinase. Therefore, p70s6k does not seem to be required for S phase entry in fibroblasts, in agreement with previous observations (26). The effects of drug treatments on earlier time points after serum stimulation were also studied (Fig. 3D). In agreement with the data shown in Fig. 3A, LY 294002 caused a profound delay in induction of cyclin D1, whereas PD 98059 causes only a slight delay.

Akt/PKB Activation Increases Levels of Endogenous Cyclin D1—The Akt/PKB kinase is believed to be activated by PI 3-kinase-generated PIP3 through a sequential mechanism. The binding of the lipid to its PH domain recruits the enzyme to the plasma membrane, where it is phosphorylated at Thr-308 and Ser-473. A kinase, termed PDK1, that can phosphorylate Thr-308 only when Akt/PKB is bound to PIP3, has been purified and cloned. PDK1 is itself dependent on PIP3 for its activity (27, 28). The identity of the Ser-473 kinase is not known at present. Thus, PIP3 is required for at least two steps in the Akt/PKB activation mechanism, the first of which can be overcome by constitutively targeting the kinase to the membrane, as is observed in its viral counterpart v-Akt, in which the viral gag sequence is myristylated. Constitutively membrane-localized Akt/PKB is thus partially, but not completely, resistant to the effects of PI 3-kinase inhibitors.

Several stable cell lines expressing gag-Akt/PKB were generated and pooled to investigate the regulation of cyclin D1 expression in the presence of enhanced Akt/PKB activity. Fig. 4A demonstrates that gag-Akt/PKB expression results in a partial rescue of cyclin D1 expression in the presence of LY 294002 (compare with Fig. 3A). At 7 h of serum treatment, LY 294002 did reduce cyclin D1 expression, but the effect was much less marked at 14 h, and by 20 h the amount of cyclin D1 was not much different to that in uninhibited cells. Intriguingly, the gag-Akt/PKB transfected cells expressed low levels of cyclin D1 in the absence of growth factors. Therefore, we determined the levels of cyclin D1 expression in individual clones expressing gag-Akt/PKB. All cell lines showed cyclin D1 expression in the absence of growth factors, albeit at lower levels than serum-starved V12 Ras-expressing cells (Fig. 4B). Thus, Akt/PKB activity may mediate some, but not all, of the effects of V12 Ras on cyclin D1 expression.

Cyclin D1 Transcription Is Controlled by Multiple Ras Effectors—Previous work on the regulation of cyclin D1 transcription has shown that the promoter is growth factor-regulated and can be activated by oncogenic mutants of Ras (16, 17, 29). As we were unable to detect any differences in cyclin D1 protein stability in the presence of LY 294002 or upon expression of activated Akt/PKB (data not shown), we investigated the regulation of cyclin D1 transcription in response to the expression of various Ras effectors. We used a reporter construct encompassing 1.8 kilobases proximal to the transcriptional start site of human cyclin D1. As shown in Fig. 5A, V12 Ras induced transcription from the reporter 4-fold. Various dominant negative alleles of Ras effectors were used to determine their contribution to this effect. Dominant negative mutants of Ral (N28 Ral) and Akt/PKB (PKB-CAA, dn Akt) both strongly reduced activated Ras induction of the cyclin D1 reporter. Al-
though activated Rac (V12 Rac), a putative PI 3-kinase target, was also able to activate the cyclin D1 reporter construct as described previously (30), a dominant negative form of the Rac (N17 Rac) gave only a minor inhibition of V12 Ras-induced transcription, suggesting that Rac does not function downstream of Ras in cyclin D1 regulation. The level of V12 Ras expression was checked and found not to vary when cells were co-transfected with dominant negative effectors. In addition, as a control, c-Jun N-terminal kinase activity was checked in transfections done in parallel to Fig. 5A: N17 Rac caused 70% inhibition of the activity of epitope tagged c-Jun N-terminal kinase-1 co-expressed with V12 Ras. This compares well with the level of inhibition of c-Jun N-terminal kinase activity by N17 Rac reported by others, indicating that the dominant negative Rac was effective on other pathways.

Activated forms of the various effectors of Ras were used to determine their effects on cyclin D1 transcription. On their own, activated PI 3-kinase as well as gag-Akt/PKB both gave signals of comparable strength to that observed upon expression of an activated form of Raf kinase (Fig. 5). Because the different Ras effectors activate distinct downstream signaling cascades, we investigated whether they would cooperate to activate cyclin D1 transcription. Another distinct Ras effector, Rlf, was also included in this analysis. Whereas expression of activated forms of Raf, Rlf, and PI 3-kinase on their own gave 2.5–3-fold inductions, the combined effect of Raf and Rlf was additive (Fig. 5B). Combined expression of PI 3-kinase and Raf as well as PI 3-kinase and Rlf synergized in cyclin D1 induction, suggesting that they use independent pathways to activate D1 transcription.

**Different Ras Effectors Can Activate E2F-dependent Transcription**—Because the presence of the PI 3-kinase inhibitor LY 294402 delayed cyclin D1 expression (Fig. 3) and Rb hyperphosphorylation (data not shown) we reasoned that the PI 3-kinase activity may be required for the release of E2F activity from Rb-mediated repression. Therefore, we used an E2F reporter derived from the adenovirus E2 promoter to assess E2F transcriptional activity (31). Fig. 6 shows that the V12 Ras-induced increase of E2F activity can be partially suppressed by co-expression of dominant-negative constructs that block the function of the Ras effectors Ral-GDS (N28 Ral) and PI 3-kinase (Ap85). Co-expressing a dominant-interfering construct ofAkt/PKB also reduced V12 Ras-dependent induction of E2F. Activated forms of PI 3-kinase and gag-Akt/PKB led to a pronounced response. The PI 3-kinase response was reduced upon co-expression of dominant-interfering Akt/PKB indicating that Akt/PKB is the predominant PI 3-kinase effector required for cell cycle progression; dominant negative Akt/PKB did not reverse the effects of activated Raf or Rlf. The observation that the Akt/PKB-mediated increase in E2F activity could be largely suppressed by co-expression of cdk inhibitor p16 suggests that PI 3-kinase acts by up-regulating cyclin D1 to affect E2F.

As dominant negative Ral (N28 Ral) was able to partially suppress the V12 Ras-induced E2F induction, activated forms of Ral as well as Rlf were also tested. L72 Ral, an activated form of Ral, only led to a minor induction. A mutant of Ral with a decreased affinity for nucleotide that retained GTPase activity, L39 Ral was also examined. The increased exchange of hydrolyzed GDP leads to a rapid reactivation of this mutant; this approach has recently been shown to lead to strong activation of hydrolyzed GDP leads to a rapid reactivation of this mutant; this approach has recently been shown to lead to strong activation of Cdc42 (32). L39 Ral led to a small but reproducible increase in E2F activity, comparable to that observed upon expression of activated Raf. These results suggest that part of the activation mediated by V12 Ras is mediated by a pathway involving Ral function, but that a greater contribution comes from the PI 3-kinase and Akt/PKB pathway.

**DISCUSSION**

Expression of oncogenic forms of Ras proteins leads to induction of cell cycle progression, causing exit of quiescent cells from G0 and passage through at least G1 and S phases in most cell types (4). In recent years, it has become clear that activated Ras proteins are capable of engaging a number of families of downstream effector proteins, thereby triggering in parallel the activation of several signaling systems, most notably the Raf/ MAP kinase pathway, the Ral-GDS pathway, and the PI 3-kinase pathway (15). The best characterized of these, the Raf/MAP kinase pathway, has been known for some time to influence the expression of cell cycle regulatory proteins, but the possible involvement of the other two pathways in cell cycle control is much less well understood. This paper explores the influence of the different Ras effector systems on cell cycle regulation, in particular attempting to determine whether the function of particular Ras pathways can be correlated with the control of different check points, or whether the activity of the different pathways is integrated at each check point.

One of the earliest changes that can been seen in the expression of cell cycle regulatory proteins following release of fibro-
blasts from quiescence using serum is an increase in the expression of cyclin D1. Expression of activated Ras will cause this in the absence of serum (8, 16, 17). At least in part, this is caused by activation of the Raf/MAP kinase pathway: in a variety of cell types, specific activation of Raf or MEK alone has been reported to stimulate expression of cyclin D1 (11, 18, 19, 33). The data in Fig. 5 show that in NIH 3T3 cells, activated Raf is able to induce cyclin D1 reporter transcription, albeit less strongly than activated Ras. Perhaps surprisingly, activated forms of the other two Ras effectors studied here, Rlf and PI 3-kinase \( \alpha \) catalytic subunit (p110), both induce cyclin D1 transcription at least as efficiently as does Raf. Furthermore, some combinations of effectors, especially those involving PI 3-kinase, appear to act synergistically. It is therefore likely that transcription of the cyclin D1 gene can be induced via a number of different pathways downstream of Ras and that high efficiency induction, particularly in response to physiological stimuli, may require the function of more than one pathway, with PI 3-kinase possibly being particularly important.

Studying endogenous cyclin D1 expression in NIH 3T3 cells released from serum starvation appears to support this hypothesis (Fig. 3). Treatment of quiescent cells with the PI 3-kinase inhibitor LY 294002 results in a considerable delay in the expression of cyclin D1 following serum stimulation. This effect is noticeable if the inhibitor is added up to 6 h after the addition of serum. By contrast, inhibition of the Raf/MAP kinase pathway by the use of the MEK inhibitor PD 98059 results in a much more modest delay, if any, in cyclin D1 expression. This indicates that the function of PI 3-kinase signaling pathways is more important in the serum regulation of cyclin D1 protein levels than is the Raf/MAP kinase pathway. Others have reported that LY 294002 inhibits the ability of insulin-like growth factor I to induce cyclin D1 expression in MCF-7 cells (34), but that PD 98059 inhibits platelet-derived growth factor-induced cyclin D1 induction in Chinese hamster embryo fibroblasts (21). However, certain caveats exist: although PD 98059 is thought to be a highly specific inhibitor of MEK, LY 294002 is known to inhibit several enzymes other than PI 3-kinase, particularly the more distant relatives of this kinase superfamily such as TOR and DNA-dependent protein kinase (35, 36). No highly specific inhibitors of PI 3-kinase have been identified. It is therefore important that the inhibitory effects of LY 294002 on cyclin D1 expression and cell cycle progression occur at doses that correlate with inhibition of PI 3-kinase activity (Fig. 2) and that the specific TOR inhibitor, rapamycin does not have strongly inhibitory effects on cyclin D1 expression (Fig. 3). As well as being regulated at the transcriptional level, cyclin D1 expression may also be controlled posttranscriptionally, allowing for other factors to influence the endogenous protein level that are not apparent in reporter transcription assays. Indeed, it has recently been reported that Akt/PKB is required for

![Fig. 5. Cyclin D1 transcription is induced by multiple Ras effectors. A, regulation of cyclin D1 transcription was analyzed by transfection of a cyclin D1 reporter construct (pGL2 D1-luciferase) along with various Ras effectors into NIH 3T3 cells. Reporter activity was analyzed 24–36 h after transfection. N28 Ral, dominant-negative Ral; dnAKT, dominant-negative AKT/PKB. B, cooperation between Ras effectors. Cyclin D1 reporter was expressed with activated forms of PI 3-kinase, Rlf, and Raf, either alone or in combination. Data are expressed as the fold induction of luciferase activity relative to the amount in cells that have been transfected only with the reporter construct under identical conditions.](image)

![Fig. 6. E2F transcriptional activity is elevated by Ras signaling pathways. Regulation of E2F activity was analyzed by transfection of a reporter containing the adenovirus E2 promoter E2F binding site along with various Ras effectors into NIH 3T3 cells. Reporter activity was analyzed 24–36 h after transfection. Data are expressed as the fold induction of luciferase activity relative to the amount in cells that have been transfected only with the reporter construct under identical conditions.](image)
up-regulation of translation of cyclin D mRNA (37) and that Akt/PKB acts through GSK-3β to stabilize cyclin D1 protein (38).

A number of signaling pathways have been characterized downstream of PI 3-kinase, including the Rho-family GTPase Rac and the serine/threonine kinase Akt/PKB. Activated forms of both these proteins are able to induce cyclin D1 reporter expression, and dominant negative Akt/PKB, and to a lesser extent dominant negative Rac, inhibits activated Ras-induced cyclin D1 expression. Rac induction of cyclin D1 transcription has been reported previously (30). There is therefore evidence that multiple pathways acting downstream of PI 3-kinase can contribute to cyclin D1 induction. When endogenous cyclin D1 levels are studied in cells stably expressing activated Ras or activated Akt/PKB, it is clear that whereas V12 Ras causes strong serum-independent expression of cyclin D1, activated Akt/PKB is capable of only a weak induction (Fig. 4), and Rlf-CAAX and V12 Rac do not cause any detectable induction (data not shown). In the case of expression of endogenous cyclin D1, multiple synergizing pathways acting downstream of Ras may therefore be particularly important, again emphasizing the possibility that regulation of cyclin D1 protein levels occurs at a number of levels in addition to the regulation of cyclin D1 gene transcription. Stable expression of gag-Akt/PKB, a membrane-localized activated form of Akt/PKB, can only partially abrogate the inhibitory effect of LY 294002: this may indicate that other pathways downstream of PI 3-kinase, such as Rac, play an important role here or that gag-Akt/PKB is not fully independent of PI 3-kinase activity. Although the need for PIP3 to translocate Akt/PKB to the membrane is supplanted by the myristylation signal on the gag fusion, gag-Akt/PKB still requires phosphorylation by the upstream kinases PDK1 and PDK2, which are at least in part dependent on PI 3-kinase activity themselves (39).

Cyclin D1 is only the earliest of the cell cycle regulators to be affected by Ras. Many other points of regulation of the cell cycle by Ras are possible at later times. A later event that is studied here is the regulation of E2F transcription factors released from Rb following its phosphorylation. When an E2F target sequence derived from the adenovirus E2 promoter was used as a reporter of E2F activity following activation of different Ras pathways, a pattern related to, but distinct from, that found for cyclin D1 transcription was seen (Fig. 6). What is especially noticeable is that activated Akt/PKB and PI 3-kinase are particularly strong inducers of E2F activity, more so than Ras itself. Because Akt/PKB and PI 3-kinase induction of cyclin D1 expression is less strong than Ras, this suggests that Akt/ PKB may act through other pathways in addition to cyclin D1 to control E2F activity. A very likely candidate is the regulation of the cyclin-dependent kinase inhibitor p27Kip1, the expression of which is suppressed following serum treatment of quiescent cells in a manner requiring Ras activity and is also down-regulated in Ras transformed cells. There is lack of agreement as to whether the Raf/MAP kinase pathway can lead to reduction in p27 expression, with some reports indicating that it can (11, 33) and others that it cannot (19). LY 294002 has been found to reduce the ability of growth factors to reduce p27 levels (40, 41), although others report that PD 98059 can inhibit the ability of activated Ras to down-regulate p27 expression (42). Further investigation will be required to determine the signaling connections between activated Ras and induction of p27 degradation.

The picture emerging of Ras regulation of the cell cycle is one in which multiple signaling pathways are activated by Ras, which then act together synergistically at several different control points. Broadly similar conclusions were reached by Yang et al. (43), using effector loop mutants of activated Ras, Ser-35, Gly-37, and Cys-40, which show some selectivity in the downstream effector pathways that they activate (14). In addition, activation of PI 3-kinase alone has been shown to promote cell cycle entry in some cell lines (44). In this scheme, it is important to distinguish between the situation in which transformed cells express a constitutively activated Ras oncoprotein and that of normal cells that use endogenous wild type Ras protein to transduce signals from various extracellular growth stimuli. Strong activation of the Raf, Ral-GDS, PI 3-kinase, Akt/ PKB, or Rac pathways by overexpression of the activated effectors, or of oncogenic Ras itself, may be sufficient to stimulate events that would not normally be activated by physiological activation of each pathway. As shown in Fig. 1, serum stimulation of NIH 3T3 cells leads to biphasic activation of endogenous Ras protein, as has been reported previously (22). Activation of endogenous MAP kinase and Akt/PKB does not correlate at all well with the time course of Ras activation; this is presumably because several other pathways are involved in the regulation of both these enzymes, including protein kinase C and calcium-dependent pathways in MAP kinase activation (45), induction of specific phosphatases in MAP kinase inactivation (46), and tyrosine kinases in the control of PI 3-kinase upstream of Akt/ PKB (47). Under normal physiological conditions, endogenous Ras is only one of several signaling molecules influencing the activities of these enzymes, although complete removal of Ras function through the expression of dominant negative Ras protein or introduction of neutralizing antibodies may cause catastrophic failure of the pathway. Similar considerations hold true for the influence of the pathways downstream of Ras on the regulation of cell cycle check points.

During normal regulation of the cell cycle, early activation of the MAP kinase pathway, in part through endogenous Ras, may initiate events leading to induction of cyclin D expression. At later points in progression through G1, other pathways also controlled in part by endogenous Ras, such as Raf-GDS, PI 3-kinase, Akt/ PKB, and Rac, may continue to provide stimulatory signals to cyclin D expression, even after MAP kinase activity has returned to basal levels. This requirement for activation of multiple pathways during normal growth regulation may ensure that a cell has been exposed to range of growth stimuli before it is able to progress through the cell cycle (48, 49). A key to the potent oncogenicity of mutant Ras is that it can activate multiple pathways continuously that may well normally contribute to cell cycle progression in a sequential manner. Multicellular organisms may guard against the oncogenic potential of Ras by mounting an anti-proliferative response to continuous strong activation of Ras; this can be in the form of induction of expression of cyclin-dependent kinase inhibitors p21Waf1/Cip1 and p16INK4a (9, 11, 12) or the induction of apoptosis in some cell types (50, 51).

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Cell Cycle Regulation by Ras Pathways

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