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Ras Links Growth Factor Signaling to the Cell Cycle Machinery via Regulation of Cyclin D1 and the Cdk Inhibitor p27^{KIP1}

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Activation of growth factor receptors by ligand binding initiates a cascade of events leading to cell growth and division. Progression through the cell cycle is controlled by cyclin-dependent protein kinases (Cdks), but the mechanisms that link growth factor signaling to the cell cycle machinery have not been established. We report here that Ras proteins play a key role in integrating mitogenic signals with cell cycle progression through G_1. Ras is required for cell cycle progression and activation of both Cdk2 and Cdk4 until ~2 h before the G_1/S transition, corresponding to the restriction point. Analysis of Cdk-cyclin complexes indicates that Ras signaling is required both for induction of cyclin D1 and for downregulation of the Cdk inhibitor p27^{KIP1}. Constitutive expression of cyclin D1 circumvents the requirement for Ras signaling in cell proliferation, indicating that regulation of cyclin D1 is a critical target of the Ras signaling cascade.

Mammalian cell proliferation is controlled by extracellular growth factors that initiate a cascade of events ultimately leading to cell growth and division. Progression through the cell cycle is then regulated by a series of cyclin-dependent protein kinases (Cdks), but the mechanisms that link growth factor signaling to the cell cycle machinery have not been established. In most cases, mitogenic growth factors act during the G_1 phase of the cell cycle, stimulating both the proliferation of quiescent cells arrested in G_0 and the progression of cycling cells through the restriction point in late G_1 (33). Progression through G_1 and the G_1/S transition in mammalian cells is regulated by Cdk4 and Cdk6, which form complexes with D-type cyclins and are activated in mid-G_1 to late G_1, and by Cdk2, which forms complexes with both cyclins D and E and is activated later in G_1 (3, 8, 25, 27, 28, 31, 40, 48, 49). One link between cell cycle progression and growth factor signaling is provided by cyclin D1, whose gene is induced as a secondary response gene following mitogenic stimulation (27, 40). Cyclin D1 is rapidly degraded, so its expression is dependent on continued growth factor stimulation until cells pass the G_1 restriction point.

In addition to dimerization with a cyclin partner, the activity of Cdks is regulated by posttranslational modifications (39, 46) and by their interactions with a family of Cdk-inhibitory proteins (41). These inhibitors roughly fall into two categories: (i) those that inhibit Cdk4 and Cdk6 only and (ii) those that inhibit several Cdks, albeit to varying degrees. The Cdk inhibitors p16^{INK4}, p15.5, p18, and p19 interact directly with Cdk4 and inhibit dimerization with cyclins (12, 16). On the other hand, p27^{KIP1}, p57^{KIP2}, and p21^{CIP1} are general Cdk inhibitors that interact with and inhibit multiple Cdk-cyclin complexes (7, 11, 13, 20, 24, 35, 37, 47, 52). These inhibitors are targeted by both growth-inhibitory and growth-stimulatory signals. For example, p21^{CIP1} is induced in response to DNA damage or serum stimulation (7, 43). p27^{KIP1} is expressed at high levels in quiescent cells and in cells arrested in G_1 by contact inhibition, transforming growth factor β, cyclic AMP, or lovastatin (14, 17, 34, 38). Conversely, some growth factors, such as interleukin-2, appear to stimulate cell proliferation at least in part by downregulating p27^{KIP1} expression (30).

While it is clear that growth factor signaling is required for progression through G_1, the intracellular signaling pathways that lead to activation of the cell cycle machinery have not been delineated. The Ras GTP-binding proteins play a central role in mitogenic signaling, acting to couple growth factor receptors to activation of a protein kinase cascade consisting of the Raf, MEK, and ERK protein kinases (23). In NIH 3T3 cells, interference with normal Ras function by injection of anti-Ras antibodies or by expression of the dominant negative mutant Ras N17 blocks both the proliferation of cycling cells and the response of quiescent (G_0) cells to growth factor stimulation (5, 9, 29). Studies using microinjection of anti-Ras antibodies have further suggested that Ras function is required not only for the G_0/G_1 transition but also for later stages of progression through G_1 (6, 29). Consistent with a possible role of Ras in cell cycle progression, several recent studies have also indicated that oncogenic Ras proteins can induce expression of cyclin D1 (1, 2, 21, 50).

In the present study, we have used the dominant negative mutant Ras N17 to analyze the role of Ras in linking growth factor signaling to activation of the cell cycle machinery. We report here that Ras activity plays a key role in integrating mitogenic signals with cell cycle progression. In particular, Ras is required both for induction of cyclin D1 and for downregulation of p27^{KIP1} until cells pass the restriction point. Constitutive expression of cyclin D1 circumvents the requirement for Ras in cell proliferation, indicating that regulation of cyclin D1 is a critical target of the Ras signaling cascade.

MATERIALS AND METHODS

Cell culture. NIH M17 cells have been previously described (5). NIH 3T3 cells and NIH M17 cells were cultured in Dulbecco's minimal essential medium with 10% bovine calf serum.

[^{3}H]thymidine incorporation. [^{3}H]thymidine was added to the cultures during the last 3 h of incubation, the medium was decanted, and the cells were washed three times with phosphate-buffered saline. One milliliter of 0.1 M NaOH containing 1 mg of yeast DNA per ml was added, and cell lysates were loaded onto glass fiber filters. The filters were washed with 10 ml of 10% trichloroacetic acid and 5 ml of ethanol, and radioactivity was measured with a scintillation counter.

Immunoprecipitation and Western blotting. Antibodies to Cdk2, Cdk4, cyclin D, cyclin E, and p21^{CIP1} were purchased from Santa Cruz (Santa Cruz, Calif.),

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Pan-Ras 10 antibody was obtained from Oncogene Sciences (Cambridge, Mass.), and antibody to $p27^{KIP1}$ was obtained from Transduction Laboratories (Lexington, Ky.). Cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 150 mM NaCl, 0.1% Nonidet P-40, 50-μg/ml phenylmethylsulfonyl fluoride, 10-μg/ml soybean trypsin inhibitor, 2-μg/ml leupeptin, 1-μg/ml aprotinin) and centrifuged. Protein concentration in supernatants was determined by Bradford assay. Supernatant protein (25 μg; 120 μg for immunoprecipitation of Ras N17) was transferred to a fresh tube and precleared with 25 μl of 50% protein A-Sepharose beads in 400 μl of immunoprecipitation buffer containing 1 μg of bovine serum albumin per ml for 1 h. The supernatants were incubated with the appropriate antibody (1 μg/sample) overnight, 10 μl of protein A-Sepharose beads was added for 1 h, and the immunocomplexes were washed with immunoprecipitation buffer thrice.

For Western blotting, 25 μg of the samples or immunocomplexes was boiled in sodium dodecyl sulfate (SDS) loading buffer for 5 min and separated by electrophoresis in SDS–10 to 15% polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked in phosphate-buffered saline containing 0.2% Tween 20 (T-PBS) and 5% nonfat dry milk, incubated with primary antibodies in T-PBS for 1 h at room temperature, washed thrice with T-PBS, and incubated with horseradish peroxidase-conjugated goat secondary antibodies (1:2,000) for 1 h. Membranes were washed four times with T-PBS, and antibody-antigen complexes were detected with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, Ill.) per the manufacturer's instructions.

Rb kinase assays. Immunocomplexes were washed twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 2.5 mM EGTA, 1 mM NaF, 100 μM $NaVO_4$, and 1 mM dithiothreitol [DTT]. Fifteen microliters of kinase buffer with 10 μCi of [γ-^{32}P]ATP and 1 μg of glutathione *S*-transferase–retinoblastoma protein (GST-Rb) was added to each tube and incubated at 30°C for 30 min (26). The reaction was stopped by addition of 10 μl of 4× SDS loading buffer. The tubes were boiled for 5 min and centrifuged, and supernatants were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylation of GST-Rb was quantified with a phosphorimager.

Assays of Cdk inhibitors. Cdk inhibitors in cell extracts were assayed by mixing experiments. Extracts of quiescent, dexamethasone-treated or serum-stimulated cells were prepared in 150 mM NaCl–0.1% Nonidet P-40–50 mM Tris-HCl (pH 7.5) (42). Serum-stimulated cell extracts (20 μg) were mixed with quiescent or dexamethasone-treated cell extract (10 to 40 μg) in a total volume of 80 μl. DTT was added to a final concentration of 1 mM, and the mixture was incubated at 30°C for 15 min and immunoprecipitated with 4 μg of anti-cyclin E antibody. Kinase activity was determined as described above.

Northern blot hybridization. Ten micrograms of total cytoplasmic RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose membranes. The membrane was baked, blocked by Denhardt solution, and hybridized with cyclin D1 probe prepared by random priming. The membrane was washed thrice and cyclin D1 mRNA was visualized with a phosphorimager.

Plasmids and transfection assays. Expression plasmids for dominant negative *ras* (*ras* N17) and *raf* (*raf*-301) mutants have previously been described (4, 9, 18). pRc/RSV-neo-D1 carrying cyclin D1 cDNA was a generous gift of Charles J. Sherr (36). NIH 3T3 cells were transfected with 50 ng of pBABE (which confers resistance to puromycin), 3 μg of Ras N17 or Raf-301 expression plasmids, and 1 μg of the cyclin D1 expression plasmid in the presence of 20 μg of calf thymus DNA as described previously (9). Cells were transferred to new dishes 3 days later in medium containing 2 μg of puromycin per ml. Puromycin-resistant colonies were stained and counted 10 days later.

RESULTS

Ras is required for cell cycle progression and Cdk activation through the restriction point. To understand the role of Ras in cell cycle progression, we initially sought to delineate the time period during which Ras is required for progression through G_1. In these experiments, we used a transfected line of NIH 3T3 cells (NIH M17 cells) in which the dominant negative Ras N17 mutant is expressed under the control of a dexamethasone-inducible promoter (5). Cells were arrested in G_0 by serum deprivation and then induced to reenter the cell cycle by serum stimulation. In the absence of dexamethasone induction, quiescent NIH M17 cells enter the S phase 11 to 12 h after serum stimulation (Fig. 1A). Cdk4 activity is low in quiescent cells and increases gradually over 4-fold 10 h after serum stimulation and 8- to 10-fold 12 to 13 h after serum stimulation (Fig. 1B). Cdk2 is activated somewhat later than Cdk4, similarly increasing about 8- to 10-fold by 12 to 13 h after serum stimulation (Fig. 1B).

To determine the time during which Ras is required for G_1 progression, parallel cultures of NIH M17 cells were treated with dexamethasone to induce Ras N17 expression at different times after serum stimulation. Induction of Ras N17 by dexamethasone addition up to 7 h after serum stimulation prevented cells from progressing to the S phase, while induction at later times was without effect (Fig. 1C). Induction of Ras N17 by addition of dexamethasone up to 7 h after serum stimulation similarly inhibited activation of Cdk4 and Cdk2 (Fig. 1D). In control experiments, dexamethasone had no effect on either DNA synthesis or the activation of Cdk2 and Cdk4 in parental NIH 3T3 cells (data not shown). Since expression of Ras N17 in these cells is first detected approximately 2 h after addition of dexamethasone (Fig. 1E), these results indicate that normal Ras signaling is required for progression through G_1 and activation of both Cdk2 and Cdk4 until approximately 9 h after serum stimulation, which is 2 h before the G_1/S transition.

To correlate this period of Ras dependency with the restriction point in late G_1 (33), we compared the effects of Ras N17 expression with growth factor withdrawal (Fig. 2). Quiescent NIH M17 cells were stimulated by addition of serum as described above. At hourly intervals, the cells were washed and cultured in serum-free medium until they were harvested 13 h after the initial serum stimulation. Serum withdrawal before 9 h after stimulation significantly inhibited progression to the S phase, whereas withdrawal at later times was without effect. These data are in agreement with previous studies (45) and indicate that passage of NIH M17 cells through the restriction point occurs approximately 9 h after serum stimulation, coinciding with the time at which progression to the S phase becomes independent of Ras signaling.

Cyclin D1 expression is dependent on Ras signaling. Since inhibition of Ras function prevented activation of Cdks during G_1 progression, we examined the expression of Cdk2, Cdk4, cyclin A, cyclin D1, and cyclin E by immunoblotting (Fig. 3). Quiescent NIH M17 cells were serum stimulated in the presence or absence of dexamethasone, and cells were harvested 0, 7, or 13 h later. Cdk2, Cdk4, and cyclin E were expressed at roughly constant levels in quiescent or serum-stimulated cells, and expression of these proteins was not affected by Ras N17. In contrast, cyclin D1 was expressed at a low level in quiescent cells and induced by serum stimulation. Induction of Ras N17 effectively inhibited cyclin D1 expression, indicating that serum induction of cyclin D1 was dependent upon Ras signaling. Expression of cyclin A was also dependent upon serum stimulation and Ras activity.

To further investigate the effects of Ras signaling on cyclin D1 synthesis and accumulation, NIH M17 cells were harvested at various times after serum addition and cell lysates were immunoblotted with anti-cyclin D1 antibodies. Cyclin D1 accumulated as early as 6 h, reaching a plateau 7 to 8 h after serum stimulation (Fig. 4A). This accumulation of cyclin D1 was abrogated if Ras N17 was induced by dexamethasone addition up to 7 h after serum stimulation but not at later times (Fig. 4B). Northern blot analysis further indicates that Ras modulates expression of cyclin D1 at the level of mRNA accumulation (Fig. 4C). In contrast to cyclin D1, cyclin D2 and cyclin D3 were undetectable in immunoblots and their mRNAs were detectable only at low levels (which were independent of Ras signaling) in Northern blots (data not shown). It therefore appears that continuous Ras signaling is required for both expression of cyclin D1 and cell cycle progression until approximately 2 h before the G_1/S transition. Interestingly, however, cyclin D1 expression appeared to become independent of Ras once cells had passed the restriction point.

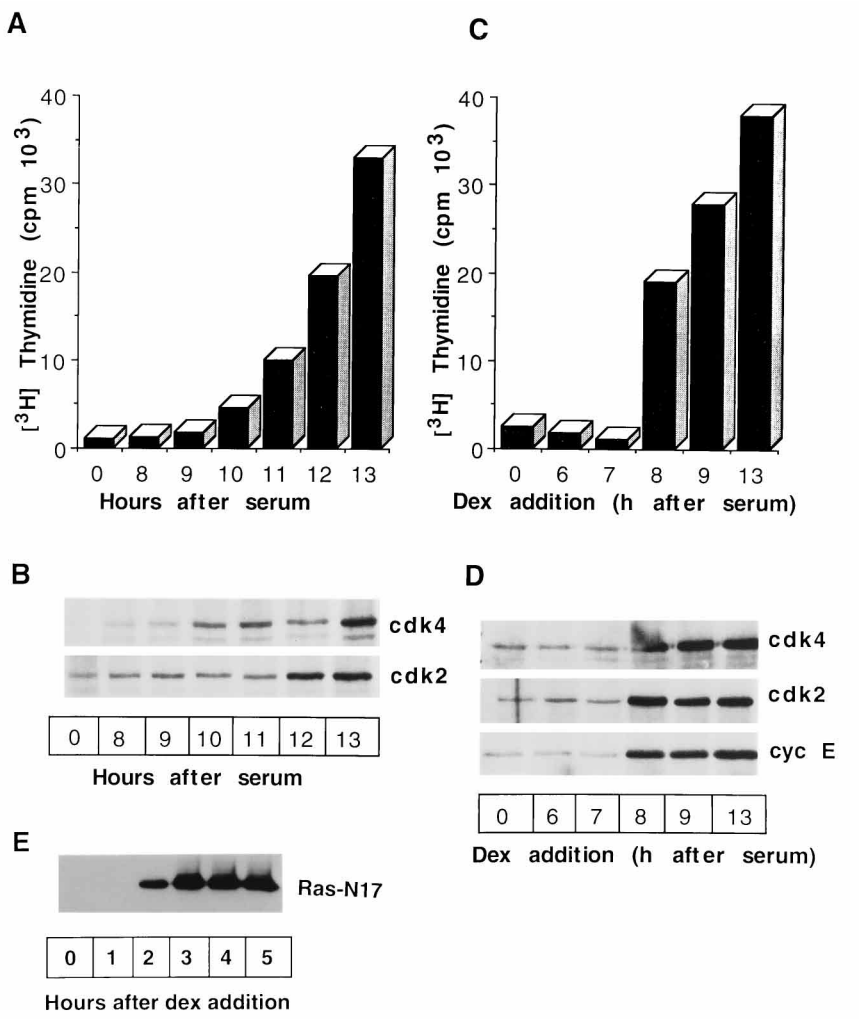

FIG. 1. Ras N17 blocks progression through G_1 and activation of Cdk2 and Cdk4 protein kinases. NIH M17 cells were synchronized by culturing in 0.2% serum for 36 h and stimulated to reenter the cell cycle by addition of 10% calf serum. Cells were harvested at the indicated times after serum stimulation (A and B). Ras N17 expression was induced by addition of dexamethasone (Dex) at the indicated times, and cells were harvested 13 h after serum stimulation (C and D). Entry into the S phase was assayed by incorporation of [^{3}H]thymidine, which was added to cultures during the last 3 h of incubation (A and C). Activities of Cdk2 and Cdk4 were determined by immunoprecipitation of parallel samples with anti-Cdk2, anti-Cdk4, and anti-cyclin E (cyc E) antibodies (B and D). Protein kinase activities of immunoprecipitates were assayed by phosphorylation of Rb. NIH M17 cells were treated with dexamethasone for the indicated times (E). Expression of Ras N17 was assayed by immunoprecipitation of 120 μg of cell extract with the anti-Ras antibody Ras-172 (10), which is specific to human Ras (from which Ras N17 is derived). Immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-Ras antibody (Pan-Ras, Oncogene Sciences).

Effect of Ras N17 on expression of p27^{KIP1} and its interaction with Cdk-cyclin complexes. The dependence of cyclin D1 accumulation on Ras signaling accounts for the inhibition of Cdk4 activity by expression of Ras N17 but not for the observed inhibition of cyclin E-dependent Cdk2 activity (Fig. 1). Since both Cdk2 and cyclin E are present at constant levels and are unaffected by Ras N17 expression, Ras must affect activity of the Cdk2-cyclin E complex by a different mechanism. A family of small proteins that interact with and inhibit activity of Cdk-cyclin complexes have recently been described. Of these inhibitors, p21^{CIP1} and p27^{KIP1} interact with both Cdk2 and Cdk4 kinases (41). The expression of p21^{CIP1} has been reported to be increased following mitogenic stimulation (43), and we have observed that p21^{CIP1} expression is induced upon serum stimulation of NIH M17 cells independently of Ras N17 expression (data not shown). On the other hand, p27^{KIP1} is expressed at high levels in resting or growth-arrested cells, and its expression is reduced by mitogenic signals, suggesting that downregulation of p27^{KIP1} might play a role in cell cycle progression (17, 34, 38). We therefore investigated the possibility that Ras signaling regulates expression of p27^{KIP1} and its interaction with different Cdk-cyclin complexes.

p27^{KIP1} was expressed at a higher level in quiescent NIH M17 cells than in cells stimulated with serum for 6 h or longer, and its expression was further reduced about threefold 10 h after serum stimulation (Fig. 5A). Consistent with previous results (32), no change in levels of p27^{KIP1} mRNA was observed (data not shown), indicating that regulation of p27^{KIP1} was posttranscriptional. Since p27^{KIP1} can associate with Cdk4-cyclin D as well as with Cdk2-cyclin E, we further sought to determine if serum stimulation and induction of cyclin D1 modified the interactions of p27^{KIP1} with different Cdk-cyclin complexes. Cell lysates were immunoprecipitated with antibodies to Cdk2 or cyclin D1, and immunoprecipitates were immunoblotted with antibodies to p27^{KIP1} (Fig. 5B). The amount of p27^{KIP1} associated with cyclin D1 increased approx-

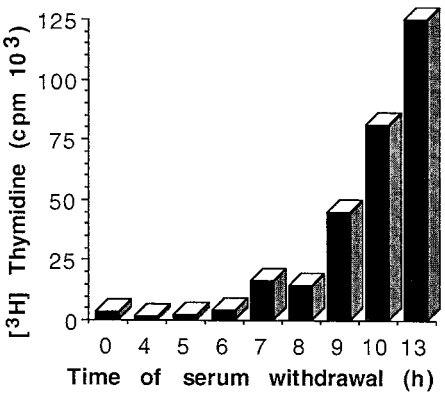

FIG. 2. Effect of serum withdrawal on cell cycle progression. Quiescent NIH M17 cells were stimulated with serum to enter the cell cycle. At the indicated times, cells were washed thrice and cultured in serum-free Dulbecco's minimal essential medium until 13 h post-initial stimulation. DNA synthesis was monitored by adding [^{3}H]thymidine to cultures in the last 3 h.

imately twofold 7 to 8 h after serum stimulation. In contrast, the amount of p27^{KIP1} associated with Cdk2 declined to low levels between 9 and 12 h after serum stimulation. These results suggest that mitogenic stimulation results in activation of Cdk2 by modulating its interaction with p27^{KIP1}. This reduced association of p27^{KIP1} with Cdk2 appears to result both from a reduction in p27^{KIP1} expression and from accumulation of cyclin D1, which shifts the association of p27^{KIP1} from Cdk2-cyclin E complexes to Cdk4-cyclin D at the time of Cdk2 activation.

These results prompted us to examine the effect of Ras N17 on expression of p27^{KIP1} and its association with Cdk-cyclin complexes. Induction of Ras N17 prior to 8 h after serum stimulation prevented the downregulation of p27^{KIP1} expression but was without effect at later times (Fig. 5C). Expression of Ras N17 similarly inhibited the increased association of p27^{KIP1} with Cdk4 or cyclin D1 (Fig. 5D), presumably as a result of inhibiting cyclin D1 accumulation. Consistent with these effects, induction of Ras N17 prior to 8 h after serum stimulation prevented the dissociation of p27^{KIP1} from Cdk2-cyclin E complexes (Fig. 5D).

Since these results suggested that maintenance of p27^{KIP1} accounted for the dependence of Cdk2 activation on Ras signaling, we further sought to determine whether the levels of p27^{KIP1} in quiescent and Ras N17-inhibited cells were sufficiently high to inhibit Cdk2 activity. Extracts of serum-stimulated cells were mixed with varying amounts of quiescent or Ras N17-inhibited cell extracts and assayed for cyclin E-dependent kinase activity. The kinase activity of serum-stimulated cell extracts was effectively inhibited by extracts of either quiescent or Ras N17-inhibited cells (Fig. 6). These cells thus contained excess Cdk inhibitor, consistent with the effects of Ras N17 on expression of p27^{KIP1} being responsible for Ras regulation of cyclin E-dependent Cdk2 activity.

Ectopic expression of cyclin D1 bypasses the Ras N17 block to cell proliferation. These data indicate that induction of cyclin D1 promotes cell cycle progression not only by activating Cdk4 but also by sequestering p27^{KIP1} and facilitating activation of Cdk2. Thus, induction of cyclin D1 might represent a primary target of Ras signaling required for cell cycle progression. To test this possibility, we asked whether constitutive expression of cyclin D1 could overcome the inhibitory effect of Ras N17 on cell proliferation (Fig. 7). As previously reported (9), cotransfection of NIH 3T3 cells with a plasmid expressing Ras N17 inhibited the outgrowth of cells transfected with a plasmid (pBABE) conferring resistance to puromycin. This inhibitory effect of Ras N17 was overcome by cotransfection with a plasmid in which cyclin D1 was constitutively expressed from a retroviral promoter. Similarly, constitutive cyclin D1 expression overcame the inhibitory effect of dominant negative Raf.

To confirm that transfection with the cyclin D1 expression plasmid allowed cotransfected cells to proliferate in the presence of Ras N17, several colonies of puromycin-resistant transformants obtained following cotransfection with plasmids expressing cyclin D1 and Ras N17 were analyzed for Ras N17 expression (Fig. 8A). The level of Ras N17 expression by these cyclin D1-cotransfected cells was similar to that observed in NIH M17 cells following induction with dexamethasone. Expression of cyclin D1 is thus sufficient to overcome a block to

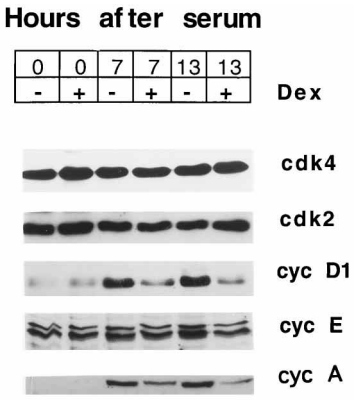

FIG. 3. Effect of Ras N17 on expression of cell cycle regulatory proteins. Quiescent NIH M17 cells were serum stimulated for 0, 7, or 13 h in the presence or absence of dexamethasone (Dex) (added at the time of serum stimulation). Expression of Cdk4, Cdk2, cyclin D1 (cyc D1), cyclin E (cyc E), and cyclin A (cyc A) was determined by immunoblotting.

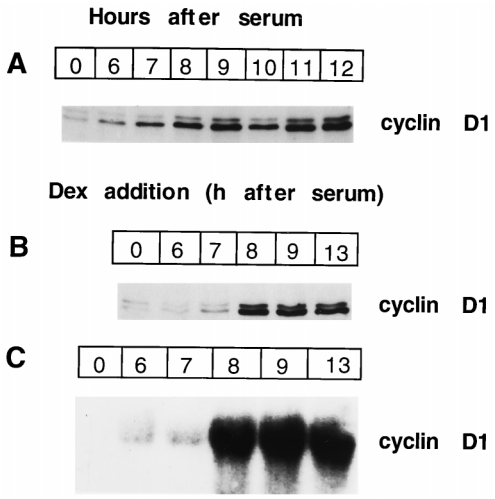

FIG. 4. Ras N17 blocks expression of cyclin D1. NIH M17 cells were harvested at the indicated times after serum stimulation, and cyclin D1 expression was determined by immunoblotting (A). Ras N17 expression was induced by addition of dexamethasone (Dex) at the indicated times (B and C). Cells were harvested 13 h after serum stimulation, and expression of cyclin D1 protein and mRNA was determined by immunoblotting (B) and Northern blotting (C), respectively.

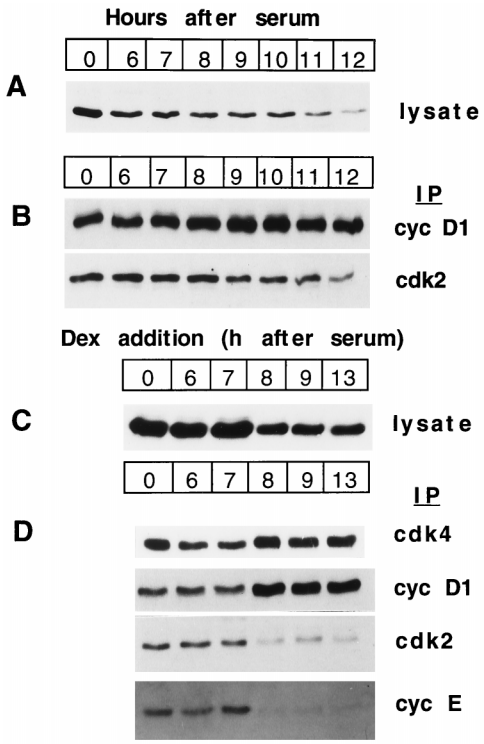

FIG. 5. Expression and association of p27^{KIP1} with Cdk-cyclin complexes. NIH M17 cells were harvested at the indicated times after stimulation (A and B). Cell lysates were immunoblotted with anti-p27^{KIP1} (A) or immunoprecipitated with anti-cyclin D1 (cyc D1) or anti-Cdk2. Immunoprecipitates (IP) were then subjected to SDS-PAGE and immunoblotted with anti-p27^{KIP1} (B). Ras N17 was induced by addition of dexamethasone (Dex) at the indicated times, and cells were harvested 13 h after serum stimulation (C and D). Expression of p27^{KIP1} was determined by immunoblotting of cell lysates (C), and the association of p27^{KIP1} with Cdk4, cyclin D, cyclin E (cyc E), and Cdk2 was determined by immunoblotting of immunoprecipitates (D).

cell proliferation resulting from inhibition of the Ras/Raf pathway, indicating that induction of cyclin D1 is a critical downstream target of Ras signaling in driving cell cycle progression.

Cyclin D1-transfected cells were further analyzed for expression of cyclin D1 and p27^{KIP1} to test the possibility that cyclin D1 regulates p27^{KIP1} expression. Three subclones of cells cotransfected with cyclin D1 plus Ras N17 and three subclones of cells transfected with cyclin D1 alone were compared to parental NIH 3T3 cells in this analysis. Cells were serum-starved and then stimulated by addition of 10% serum for 10 h, and extracts of both serum-starved and stimulated cells were analyzed for cyclin D1 and p27^{KIP1} by immunoblotting (Fig. 8B). Both parental NIH 3T3 cells and cyclin D1-transfected subclones expressed much higher levels of cyclin D1 after serum stimulation. In addition, the levels of cyclin D1 were elevated in both quiescent and serum-stimulated transfected subclones compared to the levels in the parental cells, reflecting expression of the exogenous cyclin D1 gene. Transfection with cyclin D1 did not, however, affect p27^{KIP1} expression, which was similar in both control and transfected subclones.

Further analysis of these transfected clones indicated that their doubling times varied from 16 to 21 h compared with 22 h for NIH 3T3 cells. For those clones that replicated more rapidly, the reduced cell cycle time was accounted for principally by shortening of the G_1 phase from approximately 11 h in NIH 3T3 cells to 6 to 8 h in cells overexpressing cyclin D1 (data not shown). Consistent with previous results (36), cyclin D1 overexpression thus resulted in shortening of G_1 as well as allowing Ras-independent cell proliferation. Interestingly, however, the cyclin D1-overexpressing clones remained dependent on serum for proliferation and became quiescent in low-serum medium (medium supplemented with 0.2% calf serum). Continued proliferation of these cells, while independent of Ras, therefore still required other growth factor-stimulated intracellular signaling pathways.

DISCUSSION

Ras signaling plays a key role in the mitogenic response of cells to growth factor stimulation, serving to couple receptor protein-tyrosine kinases to activation of the ERK mitogen-activated protein kinase. Although most previous studies have focused on the role of Ras in the response of quiescent cells to mitogenic stimulation, the results obtained by microinjection of anti-Ras antibodies have suggested that the requirement for normal Ras signaling is not confined to the G_0/G_1 transition but extends to mid G_1 (29). In the present study, we have confirmed and extended these observations by demonstrating that expression of dominant negative Ras N17 as late as ~2 h before initiation of the S phase, which coincides with the G_1 restriction point defined by serum withdrawal (33), is sufficient to prevent the G_1/S transition. Consistent with its effects on progression to the S phase, expression of Ras N17 inhibited the

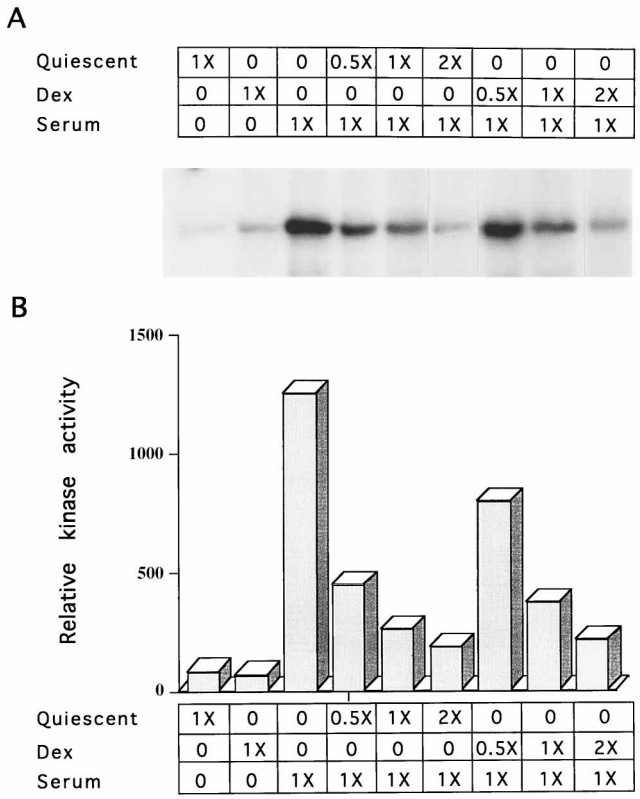

FIG. 6. Quiescent and Ras N17-expressing cells contain an inhibitor of cyclin E-dependent kinase activity. Quiescent NIH M17 cells were serum stimulated in the presence or absence of dexamethasone (to induce Ras N17) for 13 h. Extract of serum-stimulated cells (20 μg) was then mixed with 10 (0.5×), 20 (1×), or 40 μg (2×) of quiescent or dexamethasone-induced (Dex) cell extracts and incubated for 15 min at 30°C. Mixtures were then immunoprecipitated with anti-cyclin E antibody, and immunocomplexes were assayed for kinase activity by phosphorylation of Rb.

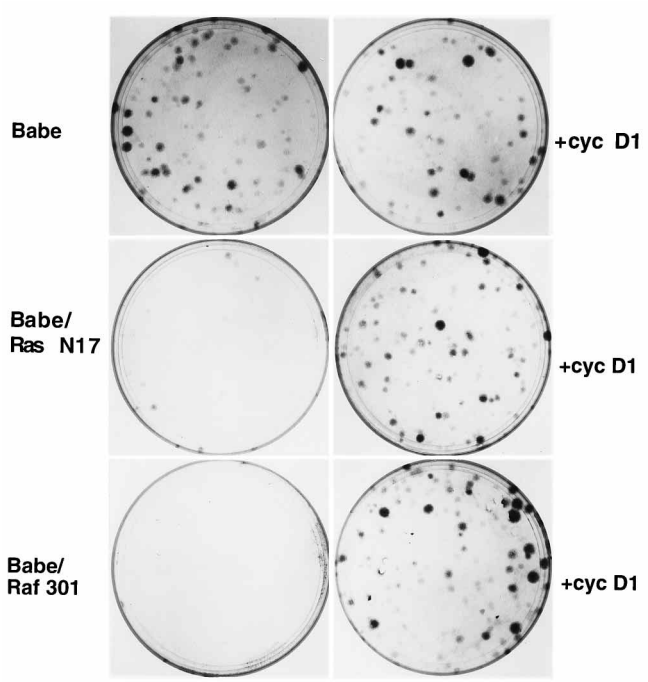

FIG. 7. Constitutive expression of cyclin D1 (cyc D1) overcomes inhibition of cell proliferation by dominant negative Ras and Raf mutants. NIH 3T3 cells were transfected with the indicated plasmid DNAs in the presence of 20 μg of calf thymus DNA. Cells were transferred to new dishes 3 days later, and transformants were selected in medium containing 2 μg of puromycin per ml. Puromycin-resistant colonies were stained 10 days later.

activities of both Cdk2 and Cdk4 during the progression of cells through G_1. It thus appears that Ras plays a key role in coupling the cell cycle machinery to growth factor stimulation.

The activities of Cdk-cyclin complexes are regulated by several mechanisms, including expression of catalytic and regulatory subunits, posttranslational modifications of catalytic subunits, and association with Cdk inhibitors. One effect of Ras N17 expression is to inhibit synthesis of cyclin D1, whose gene has been shown to be induced by mitogenic growth factors as a secondary response gene (27, 44, 51). Induction of Ras N17 either in G_0 or throughout G_1, up until the restriction point, abrogates expression of cyclin D1 at the level of mRNA accumulation. This dependence of cyclin D1 mRNA accumulation on Ras function is consistent with recent studies showing that regulatory elements of the cyclin D1 promoter are induced in response to oncogenic Ras proteins or activation of the ERK mitogen-activated protein kinase (1, 19). Taken together with the present results, it appears that one critical function of the Ras signaling pathway is induction of cyclin D1, which is required for activation of Cdk4-cyclin D complexes and passage through the restriction point (3, 27).

While regulation of cyclin D1 expression by Ras signaling accounts for inhibition of Cdk4 activity by Ras N17 expression, a similar mechanism is not responsible for inhibition of Cdk2-cyclin E complexes. Both Cdk2 and cyclin E are expressed in constant amounts in quiescent and stimulated cells, and their expression is not affected by Ras N17, indicating that the activity of Cdk2-cyclin E complexes is regulated by other mechanisms. In particular, Cdk2-cyclin E activity appears to be controlled largely by association with the Cdk inhibitor $p27^{KIP1}$. Consistent with the previously reported role of $p27^{KIP1}$ in integrating growth inhibitory signals with the cell cycle machinery (17, 34), its expression is high in quiescent cells and decreases in response to mitogenic stimulation. This decrease in intracellular level of $p27^{KIP1}$ is dependent upon Ras signaling, so that $p27^{KIP1}$ continues to be expressed at high levels when normal Ras signaling is inhibited by induction of Ras N17.

In addition to directly affecting intracellular levels of $p27^{KIP1}$, Ras signaling changes the relative amounts of $p27^{KIP1}$ associated with different Cdk-cyclin complexes as a result of induction of cyclin D1. In the absence of Ras activity, cyclin D1 is expressed at low levels, resulting in a high level of association of $p27^{KIP1}$ with Cdk2-cyclin E complexes. In contrast, normal Ras signaling results in high-level expression of cyclin D1, which increases the extent of $p27^{KIP1}$ association with Cdk4-cyclin D1 rather than Cdk2-cyclin E complexes. Thus, both the overall decrease in levels of $p27^{KIP1}$ and the increased association of $p27^{KIP1}$ with Cdk4-cyclin D1 complexes contribute to the Ras-dependent dissociation of $p27^{KIP1}$ from Cdk2-cyclin E complexes, leading to Cdk2 activation.

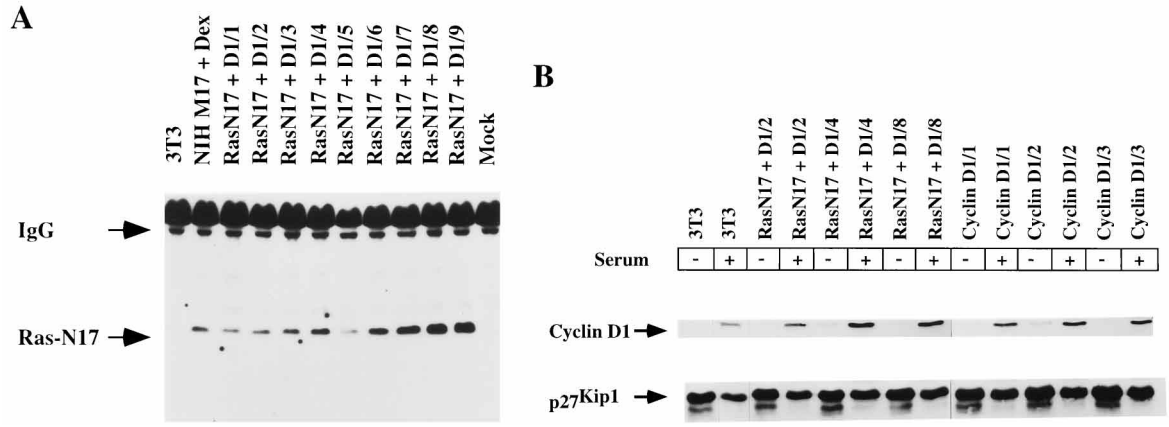

FIG. 8. Expression of Ras N17, cyclin D1, and $p27^{KIP1}$ by NIH 3T3 cells cotransfected with cyclin D1. (A) Nine independent colonies of NIH 3T3 cells cotransfected with cyclin D1 and Ras N17 (RasN17 + D1/1 to -9) were picked and cultured for analysis of Ras N17 expression, as described in the legend to Fig. 1E. Extract of NIH M17 cells induced to express Ras N17 (NIH M17 + Dex) was used as the positive control while extract of NIH 3T3 cells (3T3) was used as the negative control. (B) Quiescent or serum-stimulated parental NIH 3T3 cells (3T3), three Ras N17 + D1-cotransfected colonies (RasN17 + D1/2, -4, and -8), and three colonies transfected with cyclin D1 alone (cyclin D1/1, -2, and -3) were analyzed for expression of cyclin D1 and $p27^{KIP1}$ by immunoblotting.

The central role of cyclin D1 as a downstream target of the Ras signaling pathway is directly demonstrated by the ability of cyclin D1 overexpression to overcome the inhibition of cell proliferation resulting from expression of dominant negative Ras or Raf mutants. This effect of cyclin D1 overexpression presumably results both from its association with Cdk4 and from sequestration of $p27^{KIP1}$, leading to Cdk2 activation. Taken together, these results not only demonstrate a key role for Ras signaling in integrating the cell cycle machinery with growth factor stimulation but also indicate that regulation of cyclin D1 and $p27^{KIP1}$ are biologically critical targets of the Ras signaling pathway in controlling cell proliferation.

Although overexpression of cyclin D1 overcame the requirement for Ras signaling, it did not overcome the requirement of serum for proliferation of cyclin D1-transfected cells. This serum-dependence of cyclin D1 overexpressing cells, which is consistent with previous studies (36), indicates that both Ras-dependent and Ras-independent signaling pathways are required for cell proliferation. Cyclin D1 is thought to facilitate cell proliferation principally by promoting the phosphorylation and inactivation of Rb, and Rb-deficient cells no longer require cyclin D1 for G_1 progression (22). Importantly, however, Rb-deficient cells are still dependent upon serum (15). Taken together with the present results, these observations suggest that Ras signaling may be required principally for induction of cyclin D1 and inactivation of Rb, with other growth factor-stimulated signaling pathways serving to regulate other aspects of cell cycle progression.

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The first two authors contributed equally to this study.

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