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Ras Activity Late in G_1 Phase Required for $p27^{kip1}$ Downregulation, Passage through the Restriction Point, and Entry into S Phase in Growth Factor-Stimulated NIH 3T3 Fibroblasts

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It is well documented that Ras functions as a molecular switch for reentry into the cell cycle at the border between G_0 and G_1 by transducing extracellular growth stimuli into early G_1 mitogenic signals. In the present study, we investigated the role of Ras during the late stage of the G_1 phase by using NIH 3T3 (M17) fibroblasts in which the expression of a dominant negative Ras mutant, $p21^{Ha\text{-}Ras(Asn17)}$, is induced in response to dexamethasone treatment. We found that delaying the expression of Ras(Asn17) until late in the G_1 phase by introducing dexamethasone 3 h after the addition of epidermal growth factor (EGF) abolished the downregulation of the $p27^{kip1}$ cyclin-dependent kinase (CDK) inhibitor which normally occurred during this period, with resultant suppression of cyclin Ds/CDK4 and cyclin E/CDK2 and G_1 arrest. The immunodepletion of $p27^{kip1}$ completely eliminated the CDK inhibitor activity from EGF-stimulated, dexamethasone-treated cell lysate. The failure of $p27^{kip1}$ downregulation and G_1 arrest was also observed in cells in which Ras(Asn17) was induced after growth stimulation with a phorbol ester or α-thrombin and was mimicked by the addition late in the G_1 phase of inhibitors for phosphatidylinositol-3-kinase. Ras-mediated downregulation of $p27^{kip1}$ involved both the suppression of synthesis and the stimulation of the degradation of the protein. Unlike the earlier expression of Ras(Asn17) at the border between G_0 and G_1, its delayed expression did not compromise the EGF-stimulated transient activation of extracellular signal-regulated kinases or inhibit the stimulated expression of a principal D-type cyclin, cyclin D1, until close to the border between G_1 and S. We conclude that Ras plays temporally distinct, phase-specific roles throughout the G_1 phase and that Ras function late in G_1 is required for $p27^{kip1}$ downregulation and passage through the restriction point, a prerequisite for entry into the S phase.

When serum-deprived quiescent cells are exposed to mitogenic stimuli, including growth factors, G protein-coupled receptor agonists, and hemopoietic cytokines, cellular Ras activates within a few minutes (12, 44, 45, 54). Active Ras then interacts with and activates a number of downstream effectors, including Raf family kinases and p110 catalytic subunits of phosphatidylinositol-3-kinase (43, 55, 60). The serine and threonine protein kinase cascade consisting of Raf, MEK, and mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK]) is one of the best-characterized Ras effector systems (9). Accumulated evidence indicates that ERKs participate in the transcriptional activation of certain immediate early genes by directly phosphorylating $p62^{TCF}$/Elk-1 (13, 21), an Ets family transcription factor involved in ternary complex formation at the serum response element (19). It has also been reported recently (57) that ERKs mediate gene expression through the phosphorylation and activation of $p90^{RSK2}$, which phosphorylates CREB to activate its transactivation potential. These findings provide compelling evidence that Ras acts as a molecular switch for reentry into the cell cycle at the border between G_0 and G_1 by transducing extracellular stimuli into a number of early G_1 mitogenic signals.

By contrast, the role of Ras in later phases of the cell cycle is poorly understood. The ratio of GTP-GDP bound to Ras promptly rises after the addition of growth factors and then declines over a period of hours to a steady-state level that is slightly higher than or very close to the basal unstimulated value (31). Stacey and coworkers (11, 30) previously demonstrated that the microinjection of a neutralizing anti-Ras antibody into NIH 3T3 fibroblasts potently inhibited the initiation of DNA synthesis whether the microinjection was performed before serum stimulation or 6 h afterward. These researchers also showed that the anti-Ras antibody introduced into cells after entry into the S phase was much less inhibitory for the ongoing DNA synthesis (30). These results support the notion that the function of Ras is required for passage through the restriction (R) point late in the G_1 phase (37), the mammalian cell cycle checkpoint that is analogous to the yeast START checkpoint (16, 32), at which positive and negative cell growth regulatory signals are integrated to determine whether a cell will traverse the rest of the G_1 phase and enter the S phase. In budding yeast, genetic analysis revealed that the functions of CDC28 and three G_1-specific cyclins (*CLN1* to *CLN3*) are required for the START checkpoint (8, 32, 40). Similarly, in cultured mammalian cells, several lines of evidence indicate that the activation of G_1 cyclin-dependent kinases (CDKs), with the consequent phosphorylation and functional regulation of their substrates, including pRb and related proteins, is a prerequisite for entry into the S phase and constitutes a part of the mechanism overwhelming the R point control (2, 18, 34, 56). To date, however, besides the microinjection experiments (11, 30), there is little evidence in support of the view that Ras plays a role late in the G_1 phase to override the R point control. Also, the molecular basis for the action of Ras in the activation process of G_1 CDKs has remained elusive thus far.

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NIH 3T3 (M17) is a cell line of NIH 3T3 fibroblasts that displays an inducible expression of a dominant negative Ras, p21$^{H\text{-}Ras(Asn17)}$ [Ras(Asn17)], which is under the control of a glucocorticoid-responsive promoter sequence (5). It was previously demonstrated that dexamethasone completely inhibited epidermal growth factor (EGF)-stimulated DNA synthesis in NIH 3T3 (M17) cells but not in parental NIH 3T3 cells when it was introduced into quiescent cells 8 h before the addition of the growth factor (5). Using this method, we sought to determine whether the expression of Ras(Asn17) late in the G_1 phase exclusively inhibits entry into the S phase, and if so, how cellular Ras activity late in the G_1 phase regulates the R checkpoint control. Our results clearly indicate that the cellular Ras function is required late in the G_1 phase for the effective activation of G_1 CDKs just before the boundary between G_1 and S and for entry into the S phase. In addition, we demonstrate that Ras supports the activation of G_1 CDKs through temporally distinct mechanisms during the early and the late portions of the G_1 phase. In contrast to Ras activity early in the G_1 phase (48), Ras activity late in the G_1 phase does not affect the protein level of cyclin D1, the principal D-type cyclin required for the activation of CDK4 and CDK6 in NIH 3T3 cells. Rather, in this stage of the G_1 phase, Ras functions to regulate the protein level of the CDK inhibitor p27^{kip1}. We further demonstrate that Ras downregulates the p27^{kip1} protein level through mechanisms involving both translational and post-translational controls.

MATERIALS AND METHODS

Cell culture, cell cycle analysis, and [^{3}H]thymidine incorporation. NIH 3T3 (M17) cells, a generous gift from G. M. Cooper (Harvard Medical School), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% iron-enriched calf serum (Intergen) and 200 μg of geneticin (Sigma) at subconfluent states. Before each experiment, confluent cultures were serum deprived for 24 h in DMEM containing 0.2% bovine serum albumin (BSA) (fraction V, Sigma catalog no. A-8022). Experiments were started by the addition of mitogens at concentrations described in the figure legends, in fresh DMEM containing 0.2% BSA. For the induction of Ras(Asn17), dexamethasone (5×10^{-7} M; Sigma) was introduced into cells at various time points relative to that for the addition of mitogens (time point 0) as indicated. For cell cycle analysis, trypsinized cells were treated with the Cycle TEST DNA reagent kit (Becton Dickinson, San Jose, Calif.) according to the manufacturer's instructions. DNA fluorescence of nuclei stained with propidium iodide was measured with a FACScan flow cytometer (Becton Dickinson), and the percentages of cells within the G_0 and G_1, S, and G_2 and M phases of the cell cycle were determined. [^{3}H]thymidine incorporation into DNA was measured 18 h after the addition of mitogens as described previously (52), with [^{3}H]thymidine (1 μCi/ml; DuPont-New England Nuclear Research Products) pulse-labeled during the last hour. The data shown represent the results of more than three experiments performed in triplicate and are expressed as means ± standard errors. EGF was purchased from R&D Systems. Phorbol-12, 13-dibutyrate, α-thrombin, and thapsigargin were obtained from Sigma. The calmodulin antagonist W-7 and an inactive analog, W-5, were obtained from the Seikagaku Corporation (Tokyo, Japan). Wortmannin was obtained from Wako Chemicals. LY294002 was purchased from Carbiochem.

Immune complex kinase assay and immunoblot analysis. Immune complex kinase assays for CDK2 and CDK activating kinase (CAK) were performed by using respective rabbit polyclonal antibodies as described previously (15, 61), with histone H1 (Boehringer-Mannheim) or recombinant human CDK2 as substrates. CDK4 was immunoprecipitated from cell lysates by using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the carboxyl-terminal sequence of mouse CDK4 (FRALQHSYLHKEESDAE), and the associated kinase activity was measured with retinoblastoma protein (Rb)–glutathione *S*-transferase fusion protein as a substrate, as described previously (23). Cyclin E-CDK2 complex was immunoprecipitated by using a rabbit anti-cyclin E polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.), and associated histone H1 kinase activity was measured as described (61). In each set of experiments, cell lysates in an appropriate lysis buffer were cleared by centrifugation (10,000 × *g* at 4°C), and equal amounts of protein were subjected to immunoprecipitation. Immunoblot analysis was performed on equal amounts of cellular protein based upon protein contents determined with parallel cultures (61). For immunoprecipitation and subsequent analysis of the protein-protein interaction by immunoblotting, cell lysate was prepared in an EBC lysis buffer (23). Polyclonal rabbit anti-mouse p27^{kip1} antibodies were obtained from Santa Cruz Biotechnology and PharMingen, and the same results were obtained. To visualize p27^{kip1}, an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G Fc fragment (EY Laboratories) was employed as the secondary antibody. For immune depletion of p27^{kip1} and p21$^{Waf1/Cip1}$, rabbit polyclonal antibodies raised against cognate carboxyl-terminal peptide sequences (PharMingen and Santa Cruz Biotechnology) which specifically recognized p27^{kip1} and p21$^{Waf1/Cip1}$ were adopted. Rabbit polyclonal antibodies for cyclin A (Upstate Biotechnology Inc.), cyclin D1 (Medical and Biological Laboratories, Nagoya, Japan), and cyclins D2 and D3, and CDK6 (Santa Cruz Biotechnology) were purchased. For the detection of the CAK-mediated phosphorylation of CDK2 on Thr160, anti-CDK2 immunoprecipitate obtained from 1,800 μg of cellular protein was separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis, immunoblotted, and analyzed for specific electrophoretic mobility shift (14). The activation states of ERK1 and ERK2 were determined by electrophoretic mobility shift on immunoblot by using a mouse monoclonal anti-ERK1 and anti-ERK2 antibody (Zymed) and also by antiphosphotyrosine (4G10; Upstate Biotechnology, Inc.) immunoblot analysis, as described elsewhere (28). The results were consistent with those obtained with the anti-ERK1 and anti-ERK2 immune complex kinase assay (28) with myelin basic protein as a substrate.

Northern blot analysis. mRNA levels of cyclins A and D1 were analyzed as described in detail elsewhere (61, 62). Mouse p27^{kip1} cDNA 1 to 375 (when "A" of the initiation codon "ATG" is numbered 1) was obtained by reverse transcription-PCR amplification. After radioactive probes were stripped off the membranes, the membranes were rehybridized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, the results being used as an internal control.

Plasmids and transfections. The CDK2 expression plasmid (pME18S-CDK2) was created by ligating full-length human CDK2 cDNA (15) to the pME18S vector (a generous gift from Maruyama at Tokyo Medical and Dental School) at the *Eco*RI site downstream of the SRα promoter. A rat cyclin E expression plasmid (pcD$_2$-cyclin E) was kindly donated by H. Okayama (University of Tokyo). The rat cyclin E cDNA (−9 to 1,391) with the *Eco*RI recognition sequences added at both the 5′ and the 3′ ends was generated by PCR and ligated into pME18S at the *Eco*RI site to create pME18S-cyclin E. pSV-βgal, an expression plasmid for β-galactosidase, was purchased from Promega. The plasmids were purified by two cycles of CsCl density gradient centrifugation and introduced to cells by the calcium phosphate precipitation procedure (28).

BrdU incorporation. One day after transfection (1.2 μg of pSV-βgal and either 1.8 μg of pME18S empty vector or a combination of 0.9 μg each of pME18S-cycE and pME18S-CDK2 per 35-mm-diameter dish), the cells were serum deprived for 24 h. Quiescent cells were then growth stimulated by EGF (10 ng/ml) in fresh DMEM containing 0.2% BSA and 10 μM bromodeoxyuridine (BrdU), followed by the addition of dexamethasone 1 h later in some cultures. After 19 h, the cells were washed with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde, and permeabilized in 0.25% Triton X-100. Cells were first incubated with a rabbit polyclonal anti-β-galactosidase antibody (Cappel) and then with a rhodamine-conjugated goat anti-rabbit IgG antibody (Cappel). After fixation in 3.7% formalin and treatment in 1.5 N HCl (2), BrdU was probed sequentially with a mouse monoclonal anti-BrdU antibody (Sigma) and a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody (Zymed). Each incubation was performed according to the manufacturers' recommendations. More than 200 β-galactosidase-positive (transfected) cells were examined, and BrdU-positive cells were counted under a fluorescent microscope (Olympus, Tokyo, Japan). The expression levels of cyclin E and CDK2 in transfected cells were examined by Western blot analysis of parallel cultures and were found to be markedly elevated compared to those in empty vector-transfected controls.

^{35}S-pulse-labeling–chase experiments. Quiescent cells were first incubated for 10 h in fresh DMEM containing 0.2% BSA and EGF (10 ng/ml) to allow them to proceed toward the border between the G_1 and S phases. In some cultures, dexamethasone was added 1 h after the addition of EGF. Cells were then rinsed with warmed Dulbecco's PBS and pulse-labeled for 60 min with a mixture of [L-^{35}S]methionine and [L-^{35}S]cysteine (EXPRE^{35}S^{35}S; New England Nuclear) (100 μCi/ml) in methionine- and cysteine-deficient DMEM (Sigma) in the continued presence of EGF and dexamethasone. Labeled cells were washed twice with warmed Dulbecco's PBS and further incubated in regular DMEM containing EGF and BSA, in the presence or absence of dexamethasone. After a chase of an indicated time period, triplicate cultures were quickly washed three times with ice-cold PBS and lysed in a lysis buffer (61). To determine the extent of pulse-labeling, some cultures were washed and lyzed immediately after labeling (time point 0). Protein concentrations of the lysates were determined on parallel cultures which were treated similarly, but in the absence of ^{35}S, and 120 μg of cellular protein from each sample was taken for anti-p27^{kip1} immunoprecipitation by using a polyclonal anti-p27^{kip1} antibody (M-197; Santa Cruz Biotechnology). The immunoprecipitates were extensively washed (17), lysed in 2× Laemmli's sample buffer, and separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. After fluorography, the incorporation of ^{35}S into p27^{kip1} protein was quantitated by a Fuji-BAS 2000 Bio-Image analyzer.

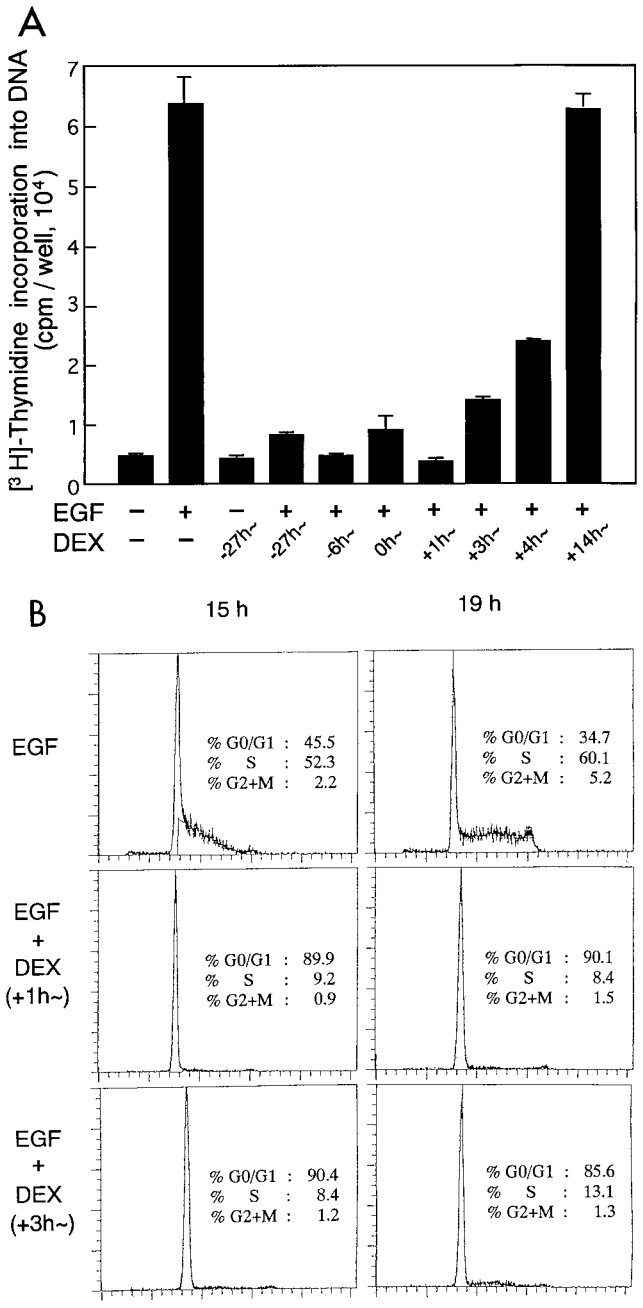

FIG. 1. (A and B) Delayed induction of Ras(Asn17) expression late in the G_1 phase prevents EGF-stimulated cells from entering the S phase. Dexamethasone (5×10^{-7} M) (DEX) was introduced to cells at time points relative to the addition of EGF (10 ng/ml at time point 0) to induce Ras(Asn17) expression. Measurements of DNA synthesis (A) and cell cycle analysis (B) were performed at 18 h (A) and at 15 and 19 h (B).

RESULTS

Delayed expression of Ras(Asn17) during the late portion of the G_1 phase causes G_1 arrest but does not interfere with a transient ERK activation in EGF-stimulated cells. We first examined whether the delayed expression of Ras(Asn17) late in the G_1 phase had any effect on G_1-to-S-phase cell cycle progression in EGF-stimulated NIH 3T3 (M17) cells (Fig. 1). We found that dexamethasone nearly completely inhibited [^{3}H]thymidine incorporation into DNA, consistently and similarly whether it was introduced into cells before or even 1 h after the addition of a maximally mitogenic concentration of EGF (Fig. 1A). The introduction of dexamethasone 3 h after the addition of EGF still potently inhibited DNA synthesis by approximately 85%, although later additions of dexamethasone were less effective. Dexamethasone had no effect when it was introduced after cells had entered the S phase (14 h after the addition of EGF). Shown in Fig. 1B are cell cycle analyses performed 15 and 19 h after the addition of EGF. Consistent with the results obtained with [^{3}H]thymidine incorporation experiments (Fig. 1A), the introduction of dexamethasone up to 3 h after the addition of EGF led to G_1 arrest in the vast majority of cells.

Previous studies demonstrated that the activation of the MEK-MAPK (ERK) pathway is necessary for mitogen-induced G_1-to-S-phase cell cycle progression (7, 36). We confirmed that the expression of a dominant negative form of either MEK1 or MAPK resulted in a potent inhibition of DNA synthesis in response to EGF and other mitogens in NIH 3T3 (M17) fibroblasts, indicating that the activation of the ERK pathway is required for mitogenesis (48). Therefore, it is possible that the delayed expression of Ras(Asn17) inhibits entry into the S phase via the inhibition of the sustained phase of ERK activation (22, 27). However, we found that the EGF-

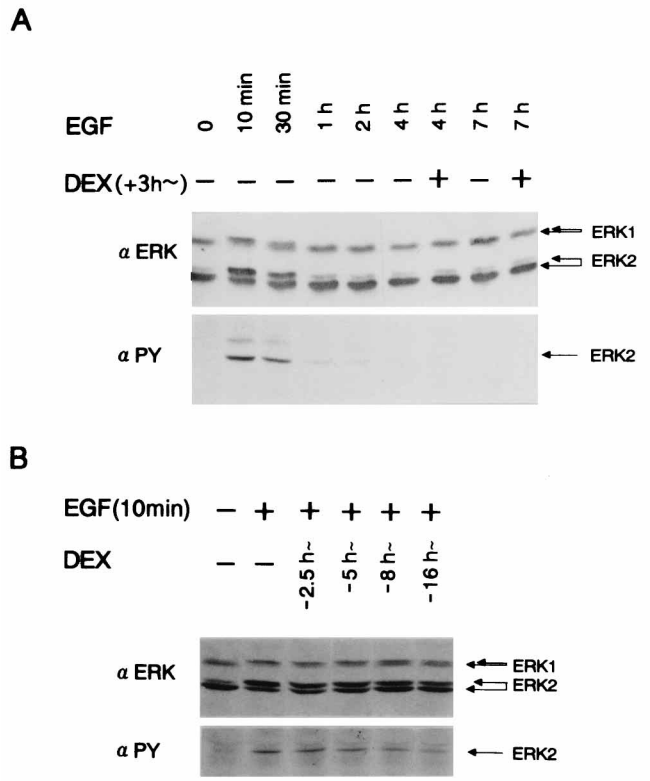

FIG. 2. (A) Temporal profile of activation of ERK1 and ERK2 in response to EGF stimulation. Quiescent cells were incubated with EGF (10 ng/ml) for the indicated periods of time, and an equal amount of protein (20 μg) was analyzed. In some cultures, dexamethasone was introduced to cells 3 h after the addition of EGF. (B) Time course of dexamethasone-induced inhibition of endogenous Ras function by Ras(Asn17) expression, as assessed by the inhibition of ERK activation. Quiescent cells were treated with dexamethasone for the indicated periods of time and then stimulated with EGF (10 ng/ml) for 10 min. The activation states of ERK1 and ERK2 were studied by band shift analysis on anti-ERK1 and anti-ERK2 immunoblots and also by antiphosphotyrosine (αPY) immunoblotting. DEX, dexamethasone.

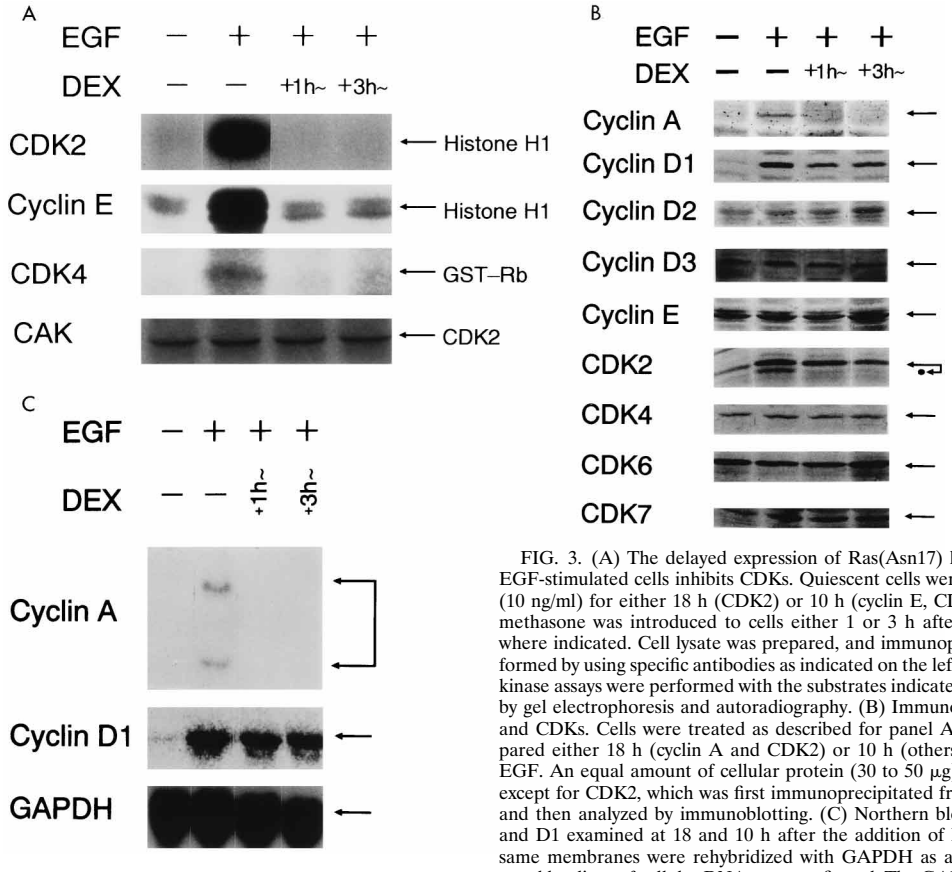

FIG. 3. (A) The delayed expression of Ras(Asn17) late in the G_1 phase of EGF-stimulated cells inhibits CDKs. Quiescent cells were stimulated with EGF (10 ng/ml) for either 18 h (CDK2) or 10 h (cyclin E, CDK4, and CAK). Dexamethasone was introduced to cells either 1 or 3 h after the addition of EGF where indicated. Cell lysate was prepared, and immunoprecipitations were performed by using specific antibodies as indicated on the left. The immune complex kinase assays were performed with the substrates indicated on the right, followed by gel electrophoresis and autoradiography. (B) Immunoblot analysis of cyclins and CDKs. Cells were treated as described for panel A, and lysates were prepared either 18 h (cyclin A and CDK2) or 10 h (others) after the addition of EGF. An equal amount of cellular protein (30 to 50 μg) was analyzed per blot except for CDK2, which was first immunoprecipitated from 1,800 μg of protein and then analyzed by immunoblotting. (C) Northern blot analysis of cyclins A and D1 examined at 18 and 10 h after the addition of EGF, respectively. The same membranes were rehybridized with GAPDH as an internal control, and equal loadings of cellular RNA were confirmed. The GAPDH result for the 10-h time point is shown. DEX, dexamethasone.

induced activation of ERKs was rather transient. As shown in Fig. 2A, the amount of the slow-migrating, tyrosine-phosphorylated active form of either p44^{ERK1} or p42^{ERK2} was maximal by 10 min, had gradually declined by 2 h, and was nearly the same as the basal unstimulated level by 4 h. The addition of dexamethasone 3 h after the EGF stimulation did not detectably affect the ERK activities measured at either 4 or 7 h poststimulation (Fig. 2A). It was previously demonstrated that the dexamethasone-induced expression of Ras(Asn17) in NIH 3T3 (M17) cells takes several hours (5). Indeed, in an examination of EGF-stimulated ERK activation (at the 10-min time point) after dexamethasone pretreatment for various time periods, the inhibition of ERK activation by dexamethasone was barely detectable until 5 h after the addition of dexamethasone (Fig. 2B). The extent of inhibition gradually increased thereafter, with a complete inhibition observed after 48 h of dexamethasone pretreatment. Thus, as estimated by the inhibition of EGF-induced ERK activation, the earliest sign of functional expression of Ras(Asn17) was detected as late as 5 h after the addition of dexamethasone. Since NIH 3T3 (M17) cells enter the S phase approximately 10 h after the addition of EGF, it is concluded that, under the conditions employed in the experiment shown in Fig. 1B, the suppression of endogenous Ras activity by Ras(Asn17) expression takes place exclusively during the late part of the G_1 phase and that this extent of the delayed Ras(Asn17) expression is sufficient to cause cell cycle arrest before the border between the G_1 and the S phases. It is also suggested that the ERK pathway does not constitute the major target of Ras action in EGF-stimulated cells during the late part of the G_1 phase, sharply contrasting with the established role of the Ras-ERK signalling cascade during the early part of the G_1 phase.

Delayed expression of Ras(Asn17) inhibits the activation of G_1 CDKs without inhibiting the expression levels of G_1 cyclins or CDKs, their associations, or CAK activity. We examined whether the selective inhibition of Ras late in the G_1 phase affected the activation of CDKs (Fig. 3A). Following the protocol adopted for Fig. 1B, dexamethasone was added to cells 1 or 3 h after the addition of EGF. Consistent with G_1 arrest (Fig. 1B), the CDK2 activity measured 18 h after the addition of EGF was completely inhibited by dexamethasone treatment. The activities of G_1 CDKs, including cyclin E/CDK2 and cyclin Ds/CDK4, were measured 10 h after the addition of EGF, which corresponds to the boundary between the G_1 and the S phases. We found in EGF-stimulated dexamethasone-treated cells that the kinase activities associated with cyclin E and CDK4 were both nearly completely inhibited to quiescent levels. By contrast, the CAK kinase activity was not affected by either EGF stimulation or Ras(Asn17) expression. Shown in Fig. 3B are immunoblot analyses of cyclin and CDK proteins studied in parallel with the kinase assay experiments (Fig. 3A). EGF stimulation for 18 h led to the expression of cyclin A, but only in the absence of the Ras(Asn17) expression. By contrast, the EGF-stimulated increase in the cyclin D1 protein level, which was maximal around the border between the G_1 and the S phases, was only minimally inhibited by the delayed expression of Ras(Asn17) (Fig. 3B). We also examined mRNA levels of cyclins A and D1 at 18 and 10 h after EGF stimulation, respectively, in the presence and absence of Ras(Asn17) ex-

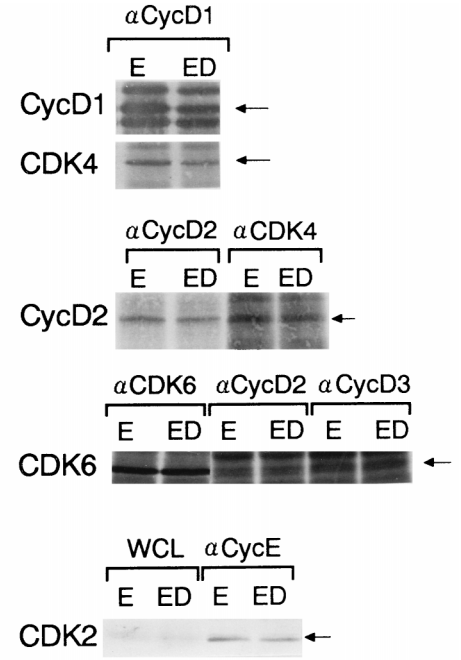

FIG. 4. Association of CDKs with partner cyclins. An equal amount of cellular protein (600 μg) from EGF-stimulated (E) or EGF-stimulated, dexamethasone-treated (ED) cells was immunoprecipitated with specific antibodies indicated at the top of each panel, followed by immunoblot analysis by probing with the antibodies as indicated at the left. WCL, whole-cell lysate (30 μg of cellular protein).

pression (Fig. 3C). The delayed Ras(Asn17) expression did not affect cyclin D1 mRNA induction at the border between G_1 and S but completely abrogated the subsequent expression of cyclin A mRNA in EGF-stimulated cells (Fig. 3C). These observations sharply contrast with our finding that the pretreatment of quiescent cells with dexamethasone prior to the addition of EGF to induce the expression of Ras(Asn17) inhibited both the mRNA and the protein levels of cyclin D1 to quiescent levels (48). As shown in Fig. 3B, the protein levels of the other G_1 cyclins, including D2, D3, and E, and those of CDK4, CDK6, and CDK7 were relatively constant until the border between G_1 and S in EGF-stimulated cells and were independent of the cellular Ras activity late in the G_1 phase. In NIH 3T3 (M17) cells, we found that cyclin E protein was relatively abundant in quiescent cells and increased after cells entered the S phase. We next studied whether the expression of Ras(Asn17) disrupts the associations between G_1 cyclin and CDK. As shown in Fig. 4, the complex formations between each of the CDKs and their partner cyclins, including cyclin D1/CDK4, cyclin D2/CDK4, cyclin D2/CDK6, cyclin D3/CDK6, and cyclin E/CDK2, were not inhibited by the delayed expression of Ras(Asn17). Consequently, the suppression of Ras activity late in the G_1 phase resulted in the inhibition of the activation of cyclin Ds/CDK4 and cyclin E/CDK2 around the boundary between G_1 and S without a reduction in the protein levels of either catalytic (CDK) or regulatory (cyclin) components or their associations and without an inhibition of the CAK activity which is responsible for activating the phosphorylation of both CDK2 and CDK4. However, the suppression of the Ras function late in the G_1 phase did inhibit the CAK-mediated phosphorylation of CDK2 at Thr160 (Fig. 3B), which is readily detected by mobility shift on gel electrophoresis (14).

Delayed expression of Ras(Asn17) prevents downregulation of $p27^{kip1}$. We next examined the possible involvement of CDK inhibitors as the target of Ras action during late G_1 phase. We found that the suppression of the endogenous Ras function of EGF-stimulated cells strongly inhibited the downregulation of $p27^{kip1}$, which normally occurs late in the G_1 phase (Fig. 5A). Thus, in EGF-stimulated cells, the protein level of $p27^{kip1}$ started to progressively decline between 3 and 6 h after the addition of the mitogen and reached a barely detectable level by 12 h. In EGF-stimulated, dexamethasone-treated cells, by contrast, $p27^{kip1}$ remained elevated for up to 16 h after the addition of EGF. It is noteworthy that the additions of dexamethasone 1 and 3 h after the addition of EGF resulted in the cessation of $p27^{kip1}$ downregulation beyond the 6- and 9-h time points, respectively (Fig. 5A). As shown in Fig. 5B, $p21^{Waf1/Cip1}$, another CDK inhibitor that also interacts with and inhibits both CDK2 and CDK4 (46), was undetectable in unstimulated cells and became detectable as a faint band after the EGF stimulation. The expression of Ras(Asn17) did not alter the expression level of $p21^{Waf1/Cip1}$. By contrast, the addition of a phorbol ester late in the G_1 phase of EGF-stimulated cells caused the upregulation of $p21^{Waf1/Cip1}$ expression (Fig. 5B) and the inhibition of progression from G_1 to S (Fig. 6B), as reported for other types of cells (59, 61).

In EGF-stimulated, dexamethasone-treated cells, the major portions of cyclins D1, D2, D3, and E were associated with $p27^{kip1}$, leaving only small fractions unassociated with $p27^{kip1}$, which remained in cell extracts after immune depletion with an anti-$p27^{kip1}$ antibody (Fig. 5C). The incubation of the arrested-cell extract derived from EGF-stimulated, dexamethasone-treated cells with the actively growing cell extract obtained just before the border between G_1 and S potently inhibited the cyclin E-associated histone H1 kinase activity in the active-cell extract (Fig. 5D). Importantly, the CDK inhibitor activity in the arrested-cell extract was completely and specifically eliminated after the immune depletion of $p27^{kip1}$ (Fig. 5D). The CDK inhibitor activity trapped in the anti-$p27^{kip1}$ immunoprecipitates was fully recovered in boiled extract of the immunoprecipitates, as reported previously (33). From these results, we conclude that the failure of the downregulation of $p27^{kip1}$ is the mechanism that inhibits the activation of the cyclin E/CDK2 complex in EGF-stimulated, Ras(Asn17)-expressing cells.

We next examined whether the enforced expression of cyclin E and CDK2 could rescue Ras(Asn17)-expressing cells from G_1 arrest and allow them to enter the S phase. As shown in Table 1, the forced expression of cyclin E and CDK2 was sufficient to stimulate quiescent cells to enter the S phase. We also found that the expression of cyclin E and CDK2 allowed EGF-stimulated dexamethasone-treated cells to enter the S phase to an extent that was comparable to that for EGF-stimulated control cells. The results are in agreement with the notion that the $p27^{kip1}$-mediated inhibition of cyclin E/CDK2 is causally related to G_1 arrest in Ras(Asn17)-expressing cells.

G_1 arrest and the failure of $p27^{kip1}$ downregulation induced by the expression of Ras(Asn17) late in the G_1 phase were not confined to EGF-stimulated cells but were also observed in cells that were growth stimulated by the G protein-coupled receptor agonist α-thrombin or the protein kinase C activator phorbol-12, 13-dibutyrate (Fig. 6A). By contrast, $p27^{kip1}$ was effectively downregulated in cells that were growth arrested by the additions of the active calmodulin antagonist W-7 (50–52), the endoplasmic reticulum Ca^{2+} pump blocker thapsigargin (49), and phorbol-12, 13-dibutyrate (61, 62) late in the G_1 phase (Fig. 6B). The results indicate that these agents apparently caused G_1 arrest through mechanisms not involving

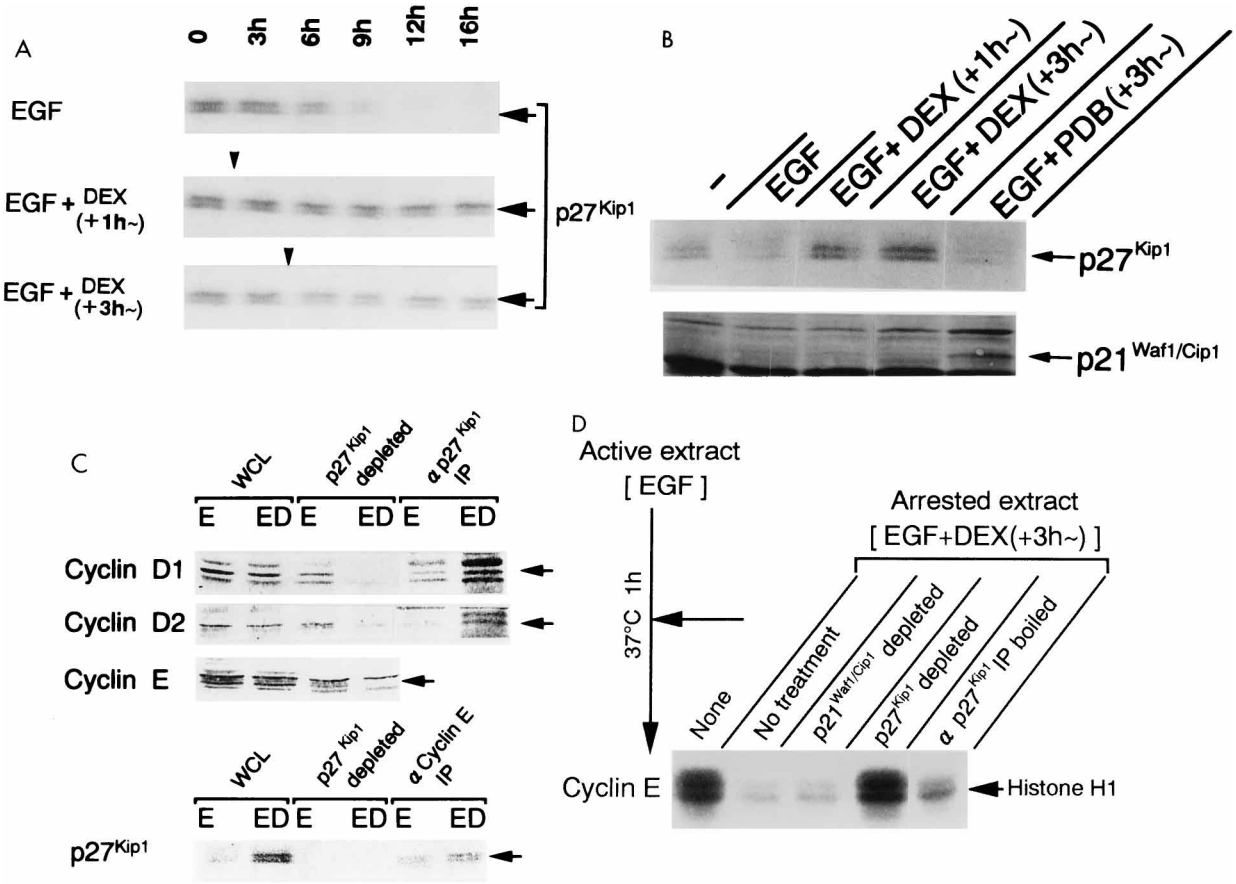

FIG. 5. (A) The delayed expression of Ras(Asn17) late in the G_1 phase prevents the downregulation of p27^{kip1} in EGF-stimulated cells. Quiescent cells were stimulated with EGF (10 ng/ml) for the indicated periods of time. Dexamethasone was introduced to cells either 1 or 3 h after the addition of EGF as indicated by the arrowheads. Thirty micrograms of cellular protein from each sample was analyzed for the level of p27^{kip1} protein by immunoblotting. (B) The delayed expression of Ras(Asn17) does not affect the protein level of p21$^{Waf1/Cip1}$. Quiescent cells were left unstimulated (−) or growth stimulated with EGF for 16 h. Dexamethasone and phorbol-12, 13-dibutyrate (PDB) (10^{-7} M) were introduced to cultures as indicated. Protein levels of p27^{kip1} and p21$^{Waf1/Cip1}$ were analyzed on 30 μg of cellular protein. (C) Sequestration of major portions of G_1 cyclins to p27^{kip1}-associated fractions in Ras(Asn17)-expressing cells. Quiescent cells were stimulated with EGF (E) or with EGF and dexamethasone added 1 h later (ED), and cell lysates were prepared 10 h after the addition of EGF. Three cycles of anti-p27^{kip1} immunoprecipitation were performed on 250 μg of cellular protein to obtain p27^{kip1}-depleted cell lysate as well as combined anti-p27^{kip1} immunoprecipitates, followed by immunoblot analysis for G_1 cyclins and p27^{kip1} as indicated on the left. The association of cyclin E with p27^{kip1} was examined by anti-p27^{kip1} immunoblotting of anti-cyclin E immunoprecipitates. The validity of anti-p27^{kip1} immunodepletion is also shown. WCL, whole-cell lysate. (D) The CDK inhibitor activity in EGF-stimulated, dexamethasone-treated cell lysate is lost after the immunodepletion of p27^{kip1}, but not p21$^{Waf1/Cip1}$, while it is recovered in anti-p27^{kip1} immunoprecipitate. Active- and arrested-cell extracts were obtained from EGF-stimulated and EGF-stimulated, dexamethasone-treated cells at 10 h and were divided into portions (660 μg of protein/tube). The arrested-cell extracts were then treated as indicated, while the active-cell extracts were kept on ice. Anti-p27^{kip1} immunoprecipitate obtained from the arrested-cell extract was boiled for 5 min, and the supernatant was recovered. After treatments, the arrested-cell extracts or a buffer control (None) were combined with the active-cell extract, and the mixture was incubated at 37°C for 1 h before an anti-cyclin E immune complex kinase assay was performed. DEX, dexamethasone; IP, immunoprecipitate.

p27^{kip1} and are consistent with the notion that the persistence of p27^{kip1} by the expression of Ras(Asn17) is the cause but not the result of G_1 arrest.

The Ras-mediated downregulation of p27^{kip1} involves regulations of both synthesis and degradation of the protein. To try to understand the mechanism by which Ras downregulates the p27^{kip1} level late in the G_1 phase, we studied whether the delayed expression of Ras(Asn17) affects the level of p27^{kip1} mRNA, the rate of p27^{kip1} protein synthesis, or degradation. As shown in Fig. 7A, Northern blot analysis revealed that the p27^{kip1} mRNA level did not change after EGF stimulation, either in the presence or the absence of dexamethasone. Shown in Fig. 7B are the results of a ^{35}S-pulse-labeling–chase experiment. Cells were treated with EGF or with EGF plus dexamethasone for 10 h, followed by ^{35}S-pulse-labeling and chase in the continued presence of EGF and dexamethasone. Most notable was the finding that the rate of synthesis of p27^{kip1} protein (as measured at time point 0 after pulse-labeling) was approximately 3.5-fold greater in dexamethasone-treated G_1-arrested cells than in EGF-stimulated cells which proceeded toward the border between G_1 and S (see Fig. 3A). In addition, the half-life of p27^{kip1} in Ras(Asn17)-expressing cells was twice as long as that of the control (5.0 versus 2.5 h). These results clearly indicate that Ras mediates the downregulation of p27^{kip1} late in the G_1 phase through regulations at posttranscriptional levels, involving both the stimulation of translation and the inhibition of degradation.

Delayed additions of wortmannin and LY294002 late in the G_1 phase prevent the downregulation of p27^{kip1} and cause G_1 arrest in mitogen-stimulated cells. Since PI3K is an established direct target of Ras action (43), we examined whether PI3K is involved as a mediator of Ras function late in the G_1

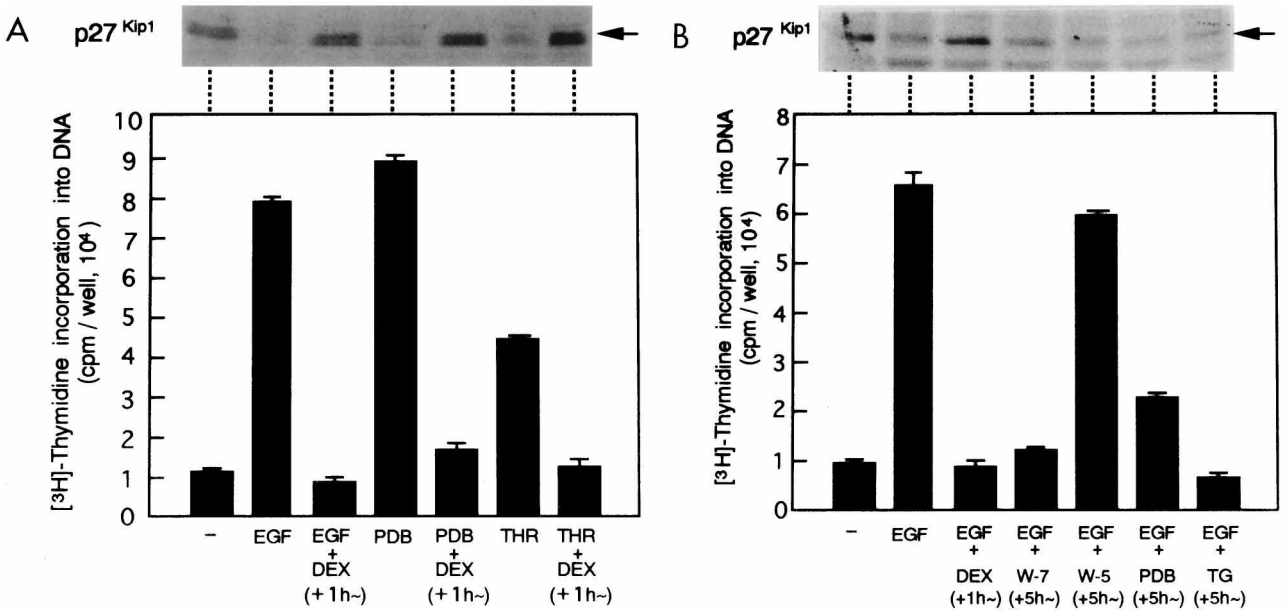

FIG. 6. (A) The delayed Ras(Asn17) expression late in the G_1 phase also causes the failure of downregulation of $p27^{kip1}$ and G_1 arrest in cells stimulated with phorbol-12, 13-dibutyrate (PDB) (10^{-7} M) or α-thrombin (THR) (3 U/ml). (B) Delayed additions (5 h after the addition of EGF) of W-7 (25 μg/ml), phorbol-12, 13-dibutyrate (10^{-7} M) or thapsigargin (TG) (10^{-7} M) cause G_1 arrest but do not affect $p27^{kip1}$ downregulation. The effects of an inactive analog, W-5, are also shown. [^{3}H]thymidine incorporation into DNA (lower panel) and $p27^{kip1}$ immunoblotting (upper panel) were performed as described in the legends for Fig. 1A and 5A, respectively.

phase. As shown in Fig. 8A, we found that the PI3K inhibitor wortmannin inhibited [^{3}H]thymidine incorporation into DNA in a manner strictly dependent on the timing of its introduction into cells relative to the addition of EGF. Thus, wortmannin (1 μM) had no effect when it was applied 15 min before the addition of EGF, whereas it potently inhibited DNA synthesis when it was introduced to EGF-stimulated cells late in the G_1 phase i.e., 4 or 7 h after the addition of EGF. When wortmannin (0.3 μM) was added at both 4 and 7 h, it nearly abolished EGF-stimulated DNA synthesis completely. Wortmannin exerted little inhibitory effect on ongoing DNA synthesis when it was applied after the border between the G_1 and S phases (14 h). Another PI3K inhibitor, LY294002 (20 μM), which is structurally unrelated to wortmannin, had a similar inhibiting effect on DNA synthesis (Fig. 8A).

Shown in Fig. 8B are the effects of wortmannin (2 μM) and LY294002 (50 μM) on the protein level of $p27^{kip1}$. Both inhibitors prevented the downregulation of $p27^{kip1}$ induced by EGF and phorbol-12, 13-dibutyrate nearly completely. These results demonstrate that cell cycle arrest induced late in the G_1 phase by wortmannin or LY294002 is accompanied by the failure of the downregulation of $p27^{kip1}$ protein, suggesting the involve-

TABLE 1. Exogenous expression of cyclin E and CDK2 induces entry into the S phase and prevents Ras(Asn17)-induced G_1 arrest[a]

Transfection	Stimulation	BrdU-positive cells (%)[b]
Empty vector	None	10.7 ± 1.3
	EGF	40.0 ± 2.8
	EGF + dexamethasone[c]	12.7 ± 1.6
Cyclin E + CDK2	None	49.9 ± 2.4
	EGF	66.2 ± 2.5
	EGF + dexamethasone[c]	64.6 ± 4.1

[a] NIH 3T3 (M17) cells were cotransfected with pSV–β-galactosidase and the expression plasmids of cyclin E and CDK2 as indicated, made quiescent, and then stimulated with EGF (10 ng/ml) for 19 h in the presence of BrdU. BrdU-positive cells in the β-galactosidase-positive cell population were counted under a fluorescent microscope.

[b] Data represent the means ± the standard errors of three determinations.

[c] Dexamethasone was added approximately 1 h after the addition of EGF.

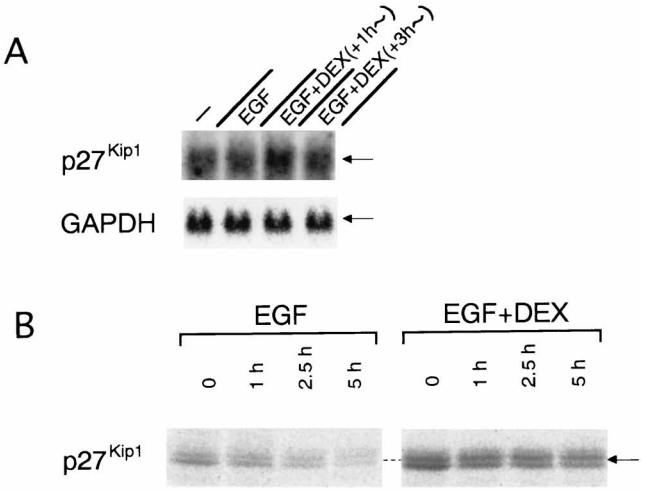

FIG. 7. (A) Northern blot analysis of $p27^{kip1}$ mRNA levels in quiescent cells (−) and in cells stimulated with EGF (10 ng/ml) for 10 h. Where indicated, dexamethasone was added to cells either 1 or 3 h after the addition of EGF. (B) ^{35}S-pulse-labeling–chase of $p27^{kip1}$ protein. Quiescent cells were first incubated for 10 h with EGF (10 ng/ml) in the presence or absence of dexamethasone, which was added 1 h after the addition of EGF. Cells were then pulse-labeled with [^{35}S]Met and [^{35}S]Cys for 60 min. At the indicated time points after pulse-labeling, cells were lysed, and 120 μg of protein was subjected to anti-$p27^{kip1}$ immunoprecipitation. The immunoprecipitates were separated by electrophoresis, followed by fluorography.

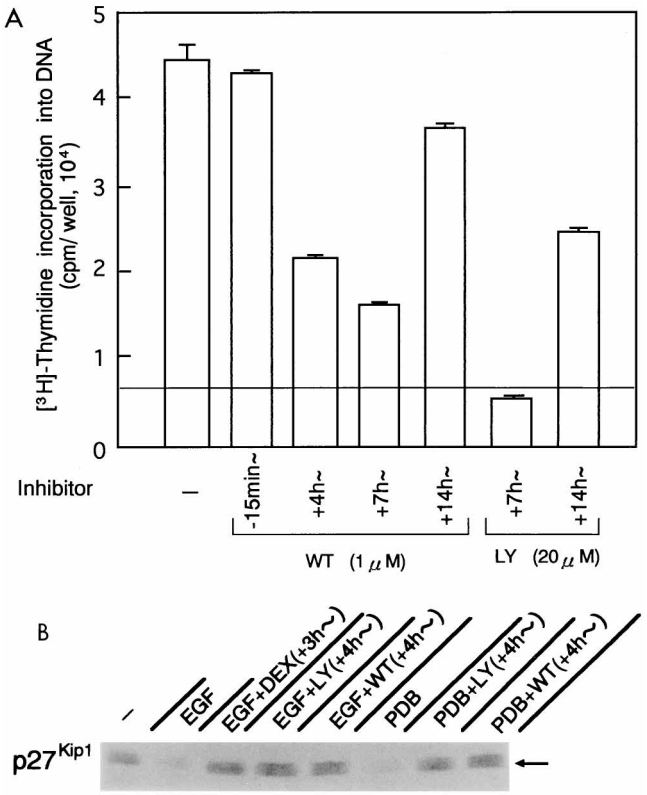

FIG. 8. (A) The additions late in the G_1 phase of wortmannin (WT) (1 μM) and LY294002 (LY) (20 μM) inhibit EGF-stimulated DNA synthesis. The mean value of the basal [^{3}H]thymidine incorporation into DNA in unstimulated cells is indicated by the horizontal line. (B) Wortmannin (2 μM) and LY29400 (50 μM) completely prevent the downregulation of p27^{kip1} when introduced into mitogen-stimulated cells late in the G_1 phase.

ment of PI3K in Ras-mediated p27^{kip1} downregulation and passage through the R point.

DISCUSSION

Cellular Ras proteins are well-characterized signalling molecules that transduce a variety of external mitogenic stimuli into the cell interior. Intensive investigation in recent years has identified ever-growing numbers of direct and indirect Ras effector molecules and established that the Ras effector signal transduction cascades contribute to the induction of diverse responses early in the G_1 phase such as the phosphorylation of cellular proteins, the generation of lipid second messengers, cytoskeletal reorganization, and gene expression. There has also been great progress in the understanding of molecular mechanisms for mammalian cell cycle transitions. It is now widely recognized that CDKs, in complex with partner cyclins, play crucial roles in the progression of cells through special phases of the cell cycle. Despite this knowledge, it is poorly understood at present how the Ras-dependent events that occur early in the G_1 phase are linked to the activation of G_1 CDKs in the subsequent stages of the cell cycle. Also, it is not firmly established whether cellular Ras activity is required late in the G_1 phase for the activation of CDKs.

In the present study, we demonstrated that the induced expression of Ras(Asn17) late in the G_1 phase caused G_1 arrest in mitogen-stimulated NIH 3T3 fibroblasts (Fig. 1 and 6A). The results are consistent with the pioneering study by Stacey and colleagues (30), who demonstrated that the microinjection of a neutralizing anti-Ras antibody late in the G_1 phase of serum-stimulated NIH 3T3 cells caused G_1 arrest. Second, we found that cellular Ras activity late in the G_1 phase determined the activation states of G_1 CDKs (Fig. 3A) without affecting the protein levels of the G_1 cyclins or CDKs (Fig. 3B), their associations (Fig. 4), or CAK activity (Fig. 3A). Third, we demonstrated that the suppression of Ras activity late in the G_1 phase caused the downregulation of the p27^{kip1} CDK inhibitor (Fig. 5A, 5B, and 6A), which normally occurred during this period, to fail, resulting in the sequestration of major portions of G_1 cyclins to p27^{kip1}-bound, inactive fractions (Fig. 5C). Immune depletion of p27^{kip1} from dexamethasone-treated, arrested-cell extract completely and specifically eliminated CDK inhibitor activity (Fig. 5D), indicating that p27^{kip1} was responsible for the inhibition of the G_1 cyclin-CDK complexes in Ras(Asn17)-expressing cells. Fourth, we found that the Ras-mediated downregulation of p27^{kip1} occurred late in the G_1 phase through mechanisms involving both the suppression of translation and the enhancement of the degradation of the protein, without a change in its mRNA level. With regard to this point, noteworthy are recent reports showing that lovastatin, which abrogates isoprenylation and plasma membrane localization of Ras, also induces the accumulation of the p27^{kip1} protein and G_1 arrest (17, 20). It was reported (17) for lovastatin-arrested HeLa cells that the rate of synthesis of the p27^{kip1} protein was three times that of asynchronous cells even though the half-life of p27^{kip1} was similar. However, it is possible that the half-life of p27^{kip1} measured at the border between G_1 and S in a synchronously proliferating population would be shorter than that determined for asynchronous cells. Fifth, the exogenous expression of cyclin E and CDK2 effectively rescued the G_1 arrest induced by the delayed expression of Ras(Asn17) (Table 1). These composite results provide compelling evidence that the function of Ras is required late in the G_1 phase for passage through the R point and that the target of Ras action during this period involves p27^{kip1}. The present study also suggests that the target of Ras action does not exist downstream of cyclin E/CDK2 activation as far as the transition from G_1 to S is concerned.

It is well documented in recent studies that the activity of each CDK is tightly controlled by multiple regulatory mechanisms, including the level of the cyclin subunit, cyclin-CDK complex formation, the phosphorylation and dephosphorylation of the CDK subunit, and the level of CDK inhibitor proteins (reference 29 and references therein). p27^{kip1} is a broad-spectrum CDK inhibitor that is capable of binding to and inhibiting cyclin Ds/CDK4, cyclin E/CDK2, cyclin A/CDK2, and cyclin B/CDC2 (39, 53; see reference 46 for a review). p27^{kip1} not only binds to and inhibits fully active cyclin-CDK complexes but also interferes physically with CAK to inhibit the CAK-mediated phosphorylation and activation of inactive cyclin-CDK complexes, whereas it does not directly inhibit CAK itself (1, 23, 46). In fact, we observed that the delayed expression of Ras(Asn17) inhibited the CAK-mediated phosphorylation of CDK2 (Fig. 3B) without reducing CAK activity itself (Fig. 3A). It was demonstrated for several cell types that growth factor stimulation of quiescent cells caused the protein level of p27^{kip1} to decline markedly during cell cycle progression through the G_1 phase via mechanisms involving both translational and posttranslational regulations (17, 23, 33, 35). Moreover, the introduction into cells of an antisense p27^{kip1} oligonucleotide (6) or the transfection of cells with an expression vector of a full-length p27^{kip1} antisense cDNA (41) led to decreased expression levels of the p27^{kip1} protein in mitogen-depleted cells and rescued these cells from

G_1 arrest. These previous observations indicate that $p27^{kip1}$ is a mediator of G_1 arrest at the R point and that the $p27^{kip1}$ protein level is the target of growth factor actions until this checkpoint is overridden. The results of the present study indicate that Ras is required for the downregulation of $p27^{kip1}$ induced by EGF as well as α-thrombin and a phorbol ester. In contrast to $p27^{kip1}$, another member of the general CDK inhibitors, $p21^{Waf1/Cip1}$, does not undergo downregulation in growth factor-stimulated cells. Interestingly, $p21^{Waf1/Cip1}$ appears to be involved in G_1 arrest induced by the addition of an active phorbol ester late in the G_1 phase (59, 61, and 62 and Fig. 5B).

Previous studies provide evidence that PI3K, a direct target of Ras (43), is required for progression from the G_1 to the S phase induced by several growth factors (42). In the present study, we found that the additions of the PI3K inhibitors late in the G_1 phase abolished the mitogen-induced downregulation of $p27^{kip1}$ and caused G_1 arrest, mimicking the effects of the delayed expression of Ras(Asn17) (Fig. 8). By contrast, the MEK inhibitor PD98059 failed to abolish mitogen-dependent $p27^{kip1}$ downregulation in NIH 3T3 (M17) cells (data not shown). Noteworthy is a report by Kimura et al. (24) showing that wortmannin exerted an analogous inhibition for the Ras-dependent phenotypic change in PC12 cells; the introduction of a single dose of wortmannin 10 h after, but not 30 min before, the addition of nerve growth factor inhibited neurite outgrowth. Given the fact that wortmannin introduced into living cells is gradually inactivated with time (24), the findings of Kimura et al. (24) and the present results (Fig. 8A) indicate that the inhibition of PI3K exclusively during late stages of growth factor activities efficiently suppresses diverse Ras-dependent cellular responses such as mitogenesis in NIH 3T3 cells (5) and the expression of the differentiated phenotype in PC12 cells (47). However, the inhibition of PI3K is not likely to be the sole mechanism for the Ras(Asn17)-mediated accumulation of $p27^{kip1}$ in NIH 3T3 (M17) cells, since, unlike the expression of Ras(Asn17), the additions of the PI3K inhibitors did not cause considerable increases in the rate of the $p27^{kip1}$ protein synthesis. Indeed, doses of the inhibitors required for the complete suppression of $p27^{kip1}$ downregulation were usually higher than those required for G_1 arrest (compare panels A and B in Fig. 8), suggesting that a mechanism other than $p27^{kip1}$ accumulation contributes to the late G_1 arrest induced by the inhibitors. Also, there are reservations about the specificity of the PI3K inhibitors. A recent study (4) demonstrated that the mammalian target of rapamycin-FKBP12 complex, mTOR (also termed FRAP/RAFT), possesses a catalytic domain homologous to that of PI3K p110 (3) and could be the direct target of both wortmannin and LY294002. With regard to this point, it should be noted that rapamycin causes the accumulation of $p27^{kip1}$ and G_1 arrest in interleukin-2-stimulated T cells (33). Evidently, further studies are required to elucidate whether PI3K or mTOR, or both, are involved in the Ras-mediated stimulation of $p27^{kip1}$ degradation and G_1 arrest as well as the molecular basis for the Ras-mediated translational control of $p27^{kip1}$.

It was previously demonstrated in NIH 3T3 (M17) cells (5) that the dexamethasone-induced expression of Ras(Asn17) prior to the addition of EGF or a phorbol ester caused G_1 arrest. This protocol of Ras(Asn17) expression should inhibit the Ras-dependent signalling events early in the G_1 phase as well as those elicited late in the G_1 phase. With this protocol also, we observed the complete inhibition of G_1 CDKs (48). However, unlike the delayed Ras(Asn17) expression protocol employed in the present study (Fig. 3B), the expression of Ras(Asn17) before EGF stimulation almost totally inhibited the induction of cyclin D1 (48). Additional studies indicated that multiple Ras targets including the MEK-ERK pathway were involved in the Ras-dependent transcriptional upregulation of cyclin D1 (48). These observations indicate that Ras is involved in the activation of G_1 CDKs in at least two ways, i.e., inducing a G_1 cyclin and downregulating the CDK inhibitor $p27^{kip1}$.

Accumulated evidence provides a model in which the R point control consists of at least two molecular events, one dependent on pRb and the other pRb independent but still dependent on serum growth factors (18). The activation of cyclin D/CDK4 or CDK6 brought about by growth factors results in the phosphorylation and inactivation of pRb (23, 46, 56), leading to the liberation of E2F1 and the consequent transcriptional activation of a series of G_1/S and S phase genes, including cyclin E (10). Thus, the activation of cyclin E/CDK2 is in part dependent on the inactivation of pRb. It was reported for Rb-null mice-derived embryo fibroblasts that the expression level of cyclin E was deregulated in such a way that the cellular content of cyclin E protein was markedly elevated in a quiescent state and superinduced by serum growth factors (through a pRb-independent mechanism) to a level 10 times higher than that in $Rb^{+/+}$ cells, resulting in the premature activation of cyclin E/CDK2 to supraphysiological extents (18). In pRb-negative cells, in which the pRb-dependent process of the R checkpoint control is lost, the expression of a CDK4- and CDK6-specific inhibitor, $p16^{INK4A}$, was shown to be ineffective in inducing G_1 arrest (26). Differently from $p16^{INK4A}$, the enforced expression of $p27^{kip1}$ resulted in G_1 arrest in pRb-negative Saos-2 cells (53), indicating that $p27^{kip1}$ also controls a pRb-independent process (other than pRb phosphorylation and inactivation). This latter action of $p27^{kip1}$ most likely involves the inhibitory control of cyclin E/CDK2, the activation of which is required if pRb-negative cells are to traverse the boundary between the G_1 and S (34). In agreement with this notion, we found that the forced expression of cyclin E/CDK2 was sufficient to overcome $p27^{kip1}$-mediated G_1 arrest induced by Ras(Asn17) (Table 1). In addition, we found that the activation of cyclin E/CDK2 in otherwise quiescent cells was capable of inducing entry into the S phase (Table 1), suggesting that pRb is a guardian of cyclin E/CDK2 activation. While the manuscript for this article was being revised, it was also demonstrated for REF52 cells that G_1 arrest induced by the adenovirus-mediated expression of Ras(Asn17) was rescued by the coexpression of cyclin E and CDK2 (25). With regard to the inhibitory control by $p27^{kip1}$ of cyclin E/CDK2, of note is a recent report (58) showing that the transcriptional activation of the cyclin A gene, which is indispensable for entry into the S phase, requires the recruitment of the active cyclin E/CDK2 complex to its promoter region via direct interaction with E2F/p107 (but not pRb) and that this recruitment is abolished by $p27^{kip1}$. Taken together, these data are consistent with the notion that Ras regulates the R point control of both pRb-dependent and -independent processes, the latter involving the relief of $p27^{kip1}$-mediated inhibition of cyclin E/CDK2. After the manuscript for this article was submitted, it was reported (38) that Ras inhibition was defective in inducing G_1 arrest in early-passage Rb-null mice-derived embryo fibroblasts. Since in those cells (18) the expression levels of cyclin E and cyclin E/CDK2 complex are so high, they likely titrate out the endogenous $p27^{kip1}$ that accumulates in response to Ras inhibition, leading to the apparent Ras-independency of R point regulation.

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