MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3370–3380 Vol. 16, No. 7
0270-7306/96/$04.00+0

Ras Induces Anchorage-Independent Growth by Subverting Multiple Adhesion-Regulated Cell Cycle Events

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Received 21 February 1996/Accepted 1 April 1996

Anchorage-independent growth is a hallmark of transformed cells, but little is known of the molecular mechanisms that underlie this phenomenon. We describe here studies of cell cycle control of anchorage-independent growth induced by the *ras* oncogene, with the use of a somatic cell mutant fibroblast line (ER-1-2) that is specifically defective in oncogene-mediated, anchorage-independent growth. Control, nontransformed PKC3-F4 cells and ER-1-2 cells cannot proliferate in semisolid medium. Three important cell cycle events are dependent on adhesion of these cells to a substratum: phosphorylation of the retinoblastoma protein, pRB; cyclin E-dependent kinase activity; and cyclin A expression. PKC3-F4 cells that express *ras* (PKC3-F4/*ras* cells) proliferate in nonadherent cultures, and each of these three events occurs in the absence of adhesion in PKC3-F4/*ras* cells. Thus, *ras* can override the adhesion requirement of cellular functions that are necessary for cell cycle progression. ER-1-2 cells that express *ras* (ER-1-2/*ras* cells) possess hyperphosphorylated forms of pRB and cyclin E-dependent kinase activity in the absence of adhesion but remain adhesion dependent for expression of cyclin A. The adhesion dependence of pRB phosphorylation and cyclin E-dependent kinase activity is therefore dissociable from the adhesion dependence of cyclin A expression. Furthermore, ectopic expression of cyclin A is sufficient to rescue anchorage-independent growth of ER-1-2/*ras* cells but does not induce anchorage-independent growth of PKC3-F4 or ER-1-2 cells. However, like pRB phosphorylation and cyclin E-dependent kinase activity, the kinase activity associated with ectopically expressed cyclin A is dependent on cell adhesion, and this dependence is overcome by *ras*. Thus, the induction of anchorage-independent growth by *ras* may involve multiple signals that lead to both expression of cyclin A and activation of G_1 cyclin-dependent kinase activities in the absence of cell adhesion.

Ras proteins play a central role in the signal transduction pathways that regulate cell proliferation and differentiation. Moreover, the H-, K-, and N-*ras* proto-oncogenes are frequent targets of somatic mutation in human cancers (3, 6, 37). Expression of an activated *ras* oncogene in cultured rodent fibroblast cell lines induces a highly pleiotropic response, including alterations in cell morphology, loss of contact inhibition, stable changes in gene expression, decreased dependence on serum growth factors, and the ability to proliferate in the absence of adhesion to a substratum (i.e., anchorage-independent growth). This last property is the best in vitro correlate of tumorigenicity (10, 17, 64). It is likely, therefore, that the molecular mechanisms that mediate anchorage-independent growth are related to the mechanisms that underlie the aggressive growth properties of naturally occurring tumors in vivo.

Most nontransformed cell types require both growth factors and cell adhesion to a substratum in order to proliferate (5, 41, 48, 51, 66). This dual requirement is thought to be due to a need for signaling events that originate from growth factor receptors and integrins, respectively. While there is overlap between receptor tyrosine kinase- and integrin-mediated signal transduction pathways (8, 22, 60, 69, 77), it appears that there may be additional, adhesion-specific events involved in mitogenesis, because high concentrations of growth factors are generally not sufficient to override the adhesion requirement. It is not clear, therefore, why mutations in certain components of growth factor signal transduction pathways (i.e., receptor tyrosine kinases and Ras, etc.) lead to an abrogation of the adhesion requirement for cell proliferation. In addition to their well-established effects on growth factor signaling pathways, it has been proposed that oncogenes might irreversibly activate pathways regulated by integrins (61). For example, adhesion can induce alkalinization of cytoplasmic pH, and certain oncogenes can mimic this effect in the absence of adhesion (62). Nevertheless, the adhesion-mediated signaling events that are responsible for the anchorage requirement in cell proliferation are unknown.

Numerous studies have highlighted the respective roles of growth factors and cell adhesion in regulating various aspects of fibroblast cell cycle progression. Soluble growth factors can stimulate adherent, G_0-arrested cells to reenter the cell cycle, and the continued presence of these growth factors is required until the late G_1-phase restriction point, R (51). After this point, adherent cells are committed to completion of a single cell cycle. Cell adhesion can influence both the G_0-to-G_1 and G_1-to-S phase transitions (11, 20, 23, 34). In the presence of serum growth factors, however, a major requirement for cell adhesion is manifested very late in G_1 phase, at a point subsequent to the majority of growth factor-mediated events (20, 23, 34). Key transitions in the mammalian cell cycle are controlled by the activation of several cyclin-dependent kinases (Cdks) (26, 27, 63). Progression through G_1 and entry into S phase require the actions of cyclins D, E, and A and their respective Cdks (26, 27, 63). Guadagno et al. have recently demonstrated that in NRK fibroblasts the expression of cyclin A, but not cyclin D or E, was dependent on cell adhesion, consistent with the failure of these cells to progress past late G_1 phase in the absence of adhesion (21). Furthermore, ectopic expression of cyclin A in NRK cells induced anchorage-independent proliferation when the cells were cultured in the presence of serum and epidermal growth factor (21). Ectopic ex-

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pression of cyclin A, however, did not cause these cells to become independent of growth factors or morphologically transformed. The relationship between these observations and anchorage-independent growth induced by oncogenes therefore remains to be explored.

To investigate the mechanism(s) by which *ras* oncogenes induce anchorage-independent growth, we have taken a somatic-cell genetic approach. A mutagen-induced variant cell line (designated ER-1-2) derived by a novel selection protocol from rat 6 embryo fibroblasts that overexpress protein kinase C β1 (PKC) has been previously isolated and characterized (see reference 32 for details). Like a control cell line (PKC3-F4), the ER-1-2 cell line continues to overexpress PKC. In striking contrast to PKC3-F4 cells, however, ER-1-2 cells fail to form colonies in soft agar when infected with a v-H-*ras*-expressing retrovirus. The ER-1-2 cell line is also resistant to anchorage-independent growth induced by the v-*src* and v-*raf* oncogenes, and the resistant phenotype is dominant in somatic-cell hybridizations (32). Despite their resistance to anchorage-independent growth, ER-1-2 cells still display transformation-related changes in morphology and gene expression in response to the *ras* oncogene (16, 32). Thus, the ER-1-2 cell line exhibits a specific defect in oncogene-induced, anchorage-independent growth. This cell system therefore permits an analysis of this aspect of oncogene action in isolation from other, less stringent parameters of the transformed phenotype. We report here a cell cycle analysis of anchorage-independent growth mediated by the v-H-*ras* oncogene in the PKC3-F4 and ER-1-2 cell lines. It has been found that at least three key cell cycle events are dependent on adhesion in these two cell lines when they do not express *ras*: phosphorylation of the retinoblastoma protein, pRB; cyclin E-dependent kinase activity; and cyclin A expression. Each of these events occurred independently of adhesion in PKC3-F4 cells expressing *ras* (PKC3-F4/*ras* cells). pRB phosphorylation and cyclin E-dependent kinase activity also occurred independently of adhesion in ER-1-2 cells that express *ras* (ER-1-2/*ras* cells), but expression of cyclin A in these cells remained adhesion dependent. Finally, ectopic expression of cyclin A rescued the ability of ER-1-2/*ras* cells to form colonies in soft agar, suggesting that expression of cyclin A in the absence of adhesion may be a key event in oncogene-mediated, anchorage-independent growth.

MATERIALS AND METHODS

Cell culture protocols. All rat 6-derived cell lines were routinely maintained in Dulbecco modified Eagle medium (DMEM) (Gibco) plus 10% bovine calf serum (Gibco), as previously described (32). NIH 3T3 and NIH 3T3/*ras* cells (provided by T. Hei and S. Kahn, Columbia University) were cultured in DMEM plus 10% fetal bovine serum. Preparative methylcellulose culture conditions and recovery of cells from such cultures were as described by Assoian et al. (1), as modified by Kume et al. (34). Briefly, 10 ml of DMEM containing 5% calf (rat 6-derived cell lines) or fetal bovine (NIH 3T3 lines) serum and 1.3% methylcellulose in a 50-ml conical tube was inoculated with 10^5 cells. The tubes were then placed in a water-jacketed CO_2 incubator at 37°C. At various times thereafter, the medium was diluted with 40 ml of ice-cold phosphate-buffered saline (to solubilize the methylcellulose), and the cells were recovered by gentle centrifugation. Recovery of cells by this method was nearly quantitative, and cell viability was >98%, as determined by trypan blue dye exclusion assays.

Soft-agar assays were performed with single wells of six-well dishes by suspending 10^4 cells in 2 ml of 0.3% Noble agar in DMEM containing 5% calf serum and overlaying the suspension on 2.5 ml of presolidified 0.5% agar in the same medium. The cultures were then overlaid with 2 ml of DMEM plus 5% calf serum and 0.3% agar every 3 or 4 days. At the end of 2 weeks, colonies were stained with the vital stain 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) for 72 h at 37°C, and colonies were counted under a low-power inverted light microscope. The colony-forming assays performed with methylcellulose (see Results) were carried out in identical fashion, except that the 0.3% top agar was replaced with 1.3% methylcellulose.

Cell synchronization. To arrest cells near the G_1/S border of the cell cycle, a double drug block was used. An initial synchronization of cells at the G_1/S border and in S phase was achieved by treating cells that were proliferating on plastic dishes with 2 mM thymidine for 14 h (30). The cells were washed twice and further incubated in fresh, standard medium for 9 h. The cultures were then either refed on the plates with medium that contained 300 μM mimosine or trypsinized and introduced into methylcellulose-containing medium that was supplemented with 300 μM mimosine. The cultures were further incubated for 16 h and then harvested for analysis. Mimosine causes cell cycle arrest very shortly before the G_1/S-phase transition (43, 70) (see Results). Over the course of several experiments, this double-block protocol resulted in 82 to 92% of cells having a 2 N DNA content as determined by fluorescence-activated cell sorter (FACS) analysis.

Flow cytometry. Cells cultured on plates or in methylcellulose-containing medium were recovered, stained with propidium iodide buffer (50 μg of propidium iodide per ml, 0.1% sodium citrate, 0.1% Triton X-100) in the presence of 10 μg of DNase-free RNase, and analyzed by flow cytometry on a FACS (FACScan; Becton Dickinson). The data were analyzed by the CellFIT software program.

Immunoblotting and in vitro cyclin E- and cyclin A-dependent kinase assays. Immunoblot analyses were performed essentially as described by Guadagno et al. (19). Cells from logarithmically growing monolayer or methylcellulose suspension cultures were harvested in lysis buffer (50 μM Tris-HCl [pH 8.0]–250 μM NaCl–1% Nonidet P-40–2 μM EDTA) containing 1 μM phenylmethylsulfonyl fluoride, 10 ng of leupeptin per ml, 50 μM NaF, and 1 μM sodium orthovanadate. Total proteins were then separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham), and the membranes were probed with specific antibodies. After extensive washing (with 40 mM Tris-HCl [pH 8.0]–50 mM NaCl–1 mM EDTA), the blots were reprobed with horseradish peroxidase-conjugated secondary antibody, and specific protein bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham). Immunoblotting was performed with the following antibodies, from the indicated sources: anti-cyclin D (06-13T), Upstate Biotechnology Inc. (UBI); anti-cyclin E, J. Roberts, Fred Hutchinson Cancer Center; anti-cyclin E (SC-481), Santa Cruz Biotechnology; anti-cyclin A, R. Assoian, University of Miami; anti-CDK2 (SC-163), Santa Cruz Biotechnology; anti-$p27^{Kip1}$ (SC-528), Santa Cruz Biotechnology; anti-human cyclin A (14531A), PharMingen; anti-human cyclin E (06-134), UBI; and anti-pRB (14001A), PharMingen.

For in vitro cyclin E- and A-dependent kinase assays, the respective cyclins were immunoprecipitated from 500 μg of total cellular protein (from lysates prepared as described above) with either polyclonal anti-cyclin E antibody (M-20; Santa Cruz Biotechnology) or monoclonal anti-human cyclin A antibody (14531A; PharMingen). The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 μM Tris-HCl [pH 7.4], 4 μM $MgCl_2$). The washed immunoprecipitates were then incubated with kinase buffer, 2 μg of histone H1, 1 μM ATP, and 5 μCi of [γ-^{32}P]ATP in a final volume of 16 μl for 30 min at 37°C. The products of the reaction were separated on an SDS–12% polyacrylamide gel. The gel was then dried and exposed to X-ray film.

Northern (RNA) blot analyses. Total cellular RNA was isolated from cells grown in monolayer cultures or methylcellulose suspension cultures, and Northern blot analyses were performed as previously described (33).

Retroviral infections. A full-length human cyclin A cDNA (35) was subcloned into the retroviral vector pBabePuro (45). Full-length cyclin D1 (28) and E (35) cDNAs were also inserted into the same vector. To generate recombinant retroviruses, the packaging mutant helper cell line ψ-2 (42) was transfected with 20 μg of either pBabePuro or pBabePuro/cyclin plasmid. Transfectants were selected in medium that contained 2.5 μg of puromycin per ml, and puromycin-resistant colonies were pooled. Viral supernatant was collected from cultures of the pooled cells and stored at −70°C. Retroviral infection of rat 6-derived cell lines was performed as previously described (32). Infected cultures were selected in medium that contained 5 μg of puromycin per ml, and resistant colonies were pooled and analyzed as described in Results.

RESULTS

Effect of adhesion on cell cycle distribution of PKC3-F4 and ER-1-2 cells with and without expression of the v-H-*ras* oncogene. The PKC3-F4 cell line overexpresses PKC but is anchorage dependent for growth in medium supplemented with 5% calf serum (75). Polyclonal pools of PKC3-F4 cells that have been infected with a high titer of v-H-*ras* retrovirus (i.e., PKC3-F4/*ras* cells) exhibit dramatic morphological alterations and form large colonies in soft-agar medium containing 5% serum (32, 75). In contrast, the ER-1-2 variant cell line also overexpresses PKC, but virus-infected ER-1-2/*ras* cells fail to proliferate in soft agar, even though they are morphologically altered (32, 75). Presumably, one or more cell cycle-regulatory events that are normally dependent on adhesion occur in the absence of adhesion when PKC3-F4 cells express the *ras* oncogene. It was of interest, therefore, to determine which cell cycle events lost adhesion dependence in these cells and which,

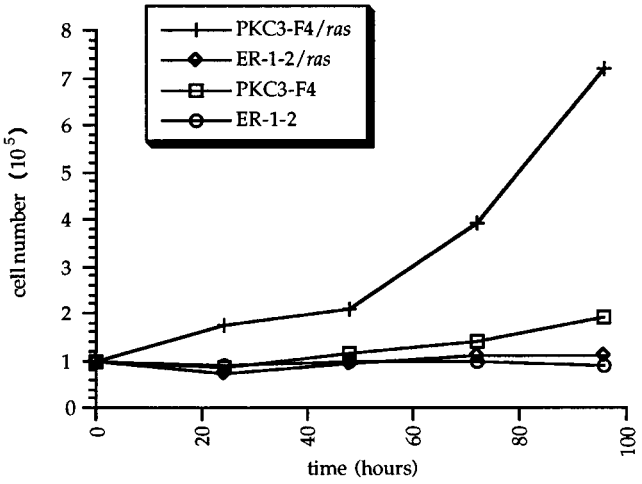

FIG. 1. Growth curves of PKC3-F4 and ER-1-2 cell lines, with and without expression of v-H-*ras*, in methylcellulose suspension cultures. Medium containing 1.3% methylcellulose was inoculated with 10^5 cells, and cells were recovered and counted at the indicated times. Points represent averages of duplicate determinations that differed by <10%. The experiment was repeated three times with similar results. See the text for details.

if any, of these events remained adhesion dependent in ER-1-2/*ras* cells.

Because it is not possible to recover viable, nonadherent cells from soft-agar cultures, we utilized a methylcellulose culture system that allows nearly quantitative recovery of intact cells (1, 34). Colony-forming assays were performed to validate the use of methylcellulose in place of soft agar. A total of 10,000 cells of each type were cultured in medium containing 1.3% methylcellulose on top of a 0.5% bottom agar layer for 2 weeks, at which time macroscopic colonies were scored. The experiment was performed twice with similar results. The colony-forming abilities of PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cells in methylcellulose culture were very similar to those observed in soft-agar cultures (32, 75); i.e., only the PKC3-F4/*ras* cells were able to form colonies ($1,570/10^4$ cells [average of triplicate determinations that differed by <10%]) under these conditions. These results are substantiated by the growth curve for each of these cell lines in preparative methylcellulose cultures, in which only PKC3-F4/*ras* cells significantly increased in number (Fig. 1).

To determine the phase of the cell cycle in which the PKC3-F4, ER-1-2, and ER-1-2/*ras* cells were arrested when deprived of adhesion, asynchronous adherent cultures were trypsinized and introduced into methylcellulose-containing medium. At various times thereafter, the cells were recovered and their cell cycle distributions were analyzed by flow cytometry. Representative results are shown in Fig. 2. Greater than 90% of the PKC3-F4 and ER-1-2 cells had arrested with a 2 N DNA content after a 48-h incubation in methylcellulose, indicating a tight arrest at some point in G_1 phase (Fig. 2A and C). PKC3-F4 and ER-1-2 cells that were in the S or G_2/M phase when placed in methylcellulose presumably completed a single cycle and subsequently arrested in G_1. The cell cycle distribution of PKC3-F4/*ras* cells reflected active growth, with cells in all phases of the cell cycle, even 48 h after loss of adhesion (Fig. 2B). ER-1-2/*ras* cells, which like PKC3-F4 and ER-1-2 cells were unable to proliferate in semisolid medium, displayed a cell cycle distribution distinct from the tight G_1 arrest observed with those two cell lines (Fig. 2D). Forty-eight hours after loss of adhesion, a significant percentage (~25%) of ER-1-2/*ras* cells remained in S phase; in fact, the profile of ER-1-2/*ras* cells was not dramatically different from that of PKC3-F4/*ras* cells, despite their inability to proliferate under these conditions. These data suggest that ER-1-2/*ras* cells arrested their growth in both G_1 and S phases when deprived of adhesion. Very similar data were obtained when two independent subclonal lines of ER-1-2/*ras* cells were analyzed in identical fashion (data not shown). Control cultures of each of the four cell types that were replated on tissue culture plastic after trypsinization, rather than introduced into methylcellulose, did not show significant alterations in their cell cycle distribution over the 48-h observation period in comparison with the initial asynchronous population (data not shown).

Effects of adhesion and *ras* on expression of cell cycle-regulatory proteins in PKC3-F4 and ER-1-2 cells. Levels of expression of cyclins D, E, and A are each rate-limiting for G_1 progression and entry into S phase (2, 18, 28, 46, 47, 50, 53–55, 57, 78). We therefore tested whether expression of any of these cyclins correlated with the flow cytometry data described above. Asynchronous, adherent cultures of PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cells were trypsinized. Methylcellulose-containing medium was inoculated with the cells, and 48 h later the cells were recovered, lysed, and analyzed by Western blotting (immunoblotting) techniques. Adherent monolayer cultures were also investigated, and the results are shown in Fig. 3A. Cyclin D1 protein levels in PKC3-F4 and ER-1-2 cells were equivalent and were not significantly affected by adhesion. Similar to data reported by others (36), expression of *ras* in these two cell lines led to an elevation of cyclin D1 levels, and this too was unaffected by the adhesive state of the cells. Cyclin E levels were similar in all four cell types, regardless of adhesive state (Fig. 3A). Cyclin A levels, however, correlated strongly with the proliferative capacity of these different cell lines in adherent and nonadherent cultures. As has been reported for NRK fibroblasts (21), cyclin A protein production was strictly anchorage dependent in both PKC3-F4 and ER-1-2 cells (Fig. 3A). ER-1-2 cells, however, displayed lower levels of cyclin A than did PKC3-F4 cells. In contrast, cyclin A levels in PKC3-F4/*ras* cells were equivalent in adherent and nonadherent cultures, consistent with the ability of these cells to proliferate in an anchorage-independent fashion. Strikingly, production of cyclin A protein in ER-1-2/*ras* cells remained almost completely adhesion dependent; only a trace amount of cyclin A was present in extracts of ER-1-2/*ras* cells that had been cultured in methylcellulose-containing medium. Consistent with these results, Northern blot analyses demonstrated that cyclin A mRNA expression paralleled cyclin A protein levels (Fig. 4).

To confirm these results, we analyzed cyclin A protein levels in adherent and nonadherent cultures that had been arrested near the G_1/S border via drug treatment. A protocol based on a double, thymidine-mimosine block was developed so that even the highly transformed PKC3-F4/*ras* cells could be arrested at this point in the cell cycle (see Materials and Methods for details). The initial thymidine block was performed while the cells were adherent, and the subsequent mimosine block could be achieved in either adherent or nonadherent cultures. Mimosine arrests cells in the narrow window of time subsequent to cyclin A synthesis but prior to the beginning of S phase (43, 70). Figure 3B demonstrates that cyclin A production in the drug-arrested cultures closely mirrored that observed in untreated cultures (Fig. 3A). Production of cyclin A protein was strictly adhesion dependent in PKC3-F4 and ER-1-2 cells and was adhesion independent in PKC3-F4/*ras* cells but remained almost completely adhesion dependent in ER-1-2/*ras* cells.

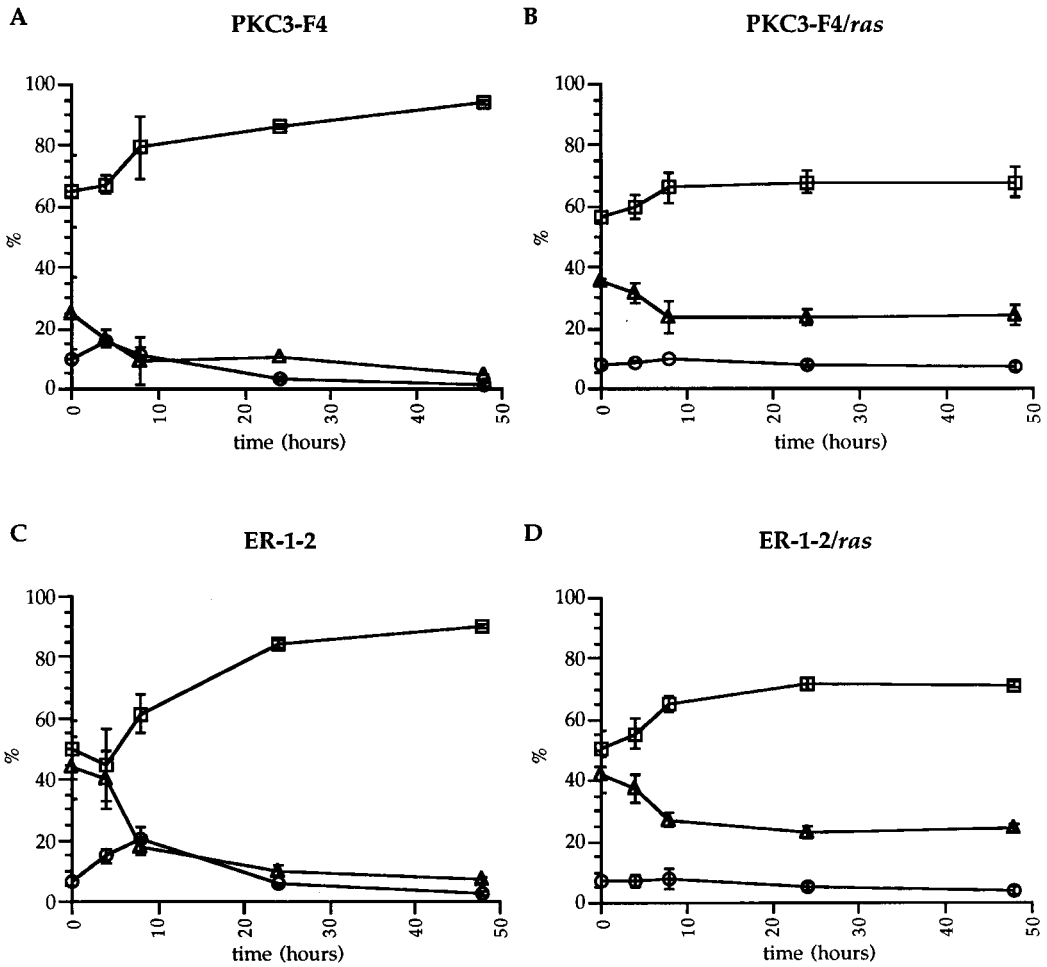

FIG. 2. Flow cytometric analysis of cell cycle progression in methylcellulose cultures. Cells growing logarithmically on cell culture plates were harvested by trypsinization, and medium containing 1.3% methylcellulose was inoculated with 10^4 cells per ml. Cells were recovered at the indicated times, stained with propidium iodide, and analyzed by flow cytometry, as described in Materials and Methods. (A) PKC3-F4 cells; (B) PKC3-F4/*ras* cells; (C) ER-1-2 cells; (D) ER-1-2/*ras* cells. Points represent mean values from three independent experiments ± 1 standard deviation. □, G_1 phase; △, S phase; ○, G_2 and M phases.

The data described above suggested that the ability to direct expression of cyclin A in the absence of adhesion could be a crucial aspect of *ras*-mediated, anchorage-independent growth. To investigate this hypothesis further, we analyzed the expression levels of proteins that mediate or influence cyclin A function. Cyclin A presumably exerts its effects on the G_1-to-S phase transition via activation of its catalytic partner, Cdk2 (13, 49, 58, 68). It was of interest, therefore, to determine whether Cdk2 levels were affected by adhesion or expression of *ras*. Figure 3A demonstrates that the amounts of Cdk2 protein produced in PKC3-F4 and PKC3-F4/*ras* cells were similar, regardless of adhesive state. In contrast, loss of adhesion resulted in a modest decrease in Cdk2 levels in ER-1-2 and ER-1-2/*ras* cells. Thus, Cdk2 production did not correlate with proliferative capacity in this system; i.e., although adhesion played a small role in regulating Cdk2 levels in the variant cells, there was no significant difference in the levels of this protein in PKC3-F4 and PKC3-F4/*ras* cells. Furthermore, the amount of Cdk2 produced by ER-1-2/*ras* cells under nonadherent conditions was apparently sufficient for cell cycle progression, once these cells were provided with cyclin A (see below). We also investigated the expression of two Cdk inhibitors that are capable of influencing G_1-phase progression and can inhibit cyclin A-Cdk2 enzyme activity: $p27^{Kip1}$ (52, 67) and $p21^{Cip1/WAF1}$ (12, 24, 73). $p27^{Kip1}$ was relatively abundant in both PKC3-F4 and ER-1-2 cells, and adhesion had no effect on its expression in these cell types (Fig. 3C; the larger quantity of $p27^{Kip1}$ observed in adherent PKC3-F4 cells than in nonadherent cells in this particular experiment was not reproducible). In contrast, PKC3-F4/*ras* and ER-1-2/*ras* cells displayed substantially decreased levels of $p27^{Kip1}$ expression, again regardless of their adhesive state. Thus, $p27^{Kip1}$ expression appeared to be a target of Ras signals but did not correlate directly with anchorage-independent cell proliferation in this system. We were unable to detect any expression of $p21^{Cip1/WAF1}$ at the protein or RNA level in any of these cell types under any conditions (data not shown).

Regulation of pRB phosphorylation and cyclin E-dependent kinase activity by adhesion is supplanted by expression of *ras*. The adhesion dependence of cyclin A expression may arise because the cyclin A gene is a direct target of adhesion-mediated signaling pathways. Alternatively, cyclin A expression might not be directly regulated by adhesion-mediated signals but could be dependent on the successful completion of an earlier cell cycle event that is itself directly regulated by adhesion; once this earlier event has occurred, cyclin A expression

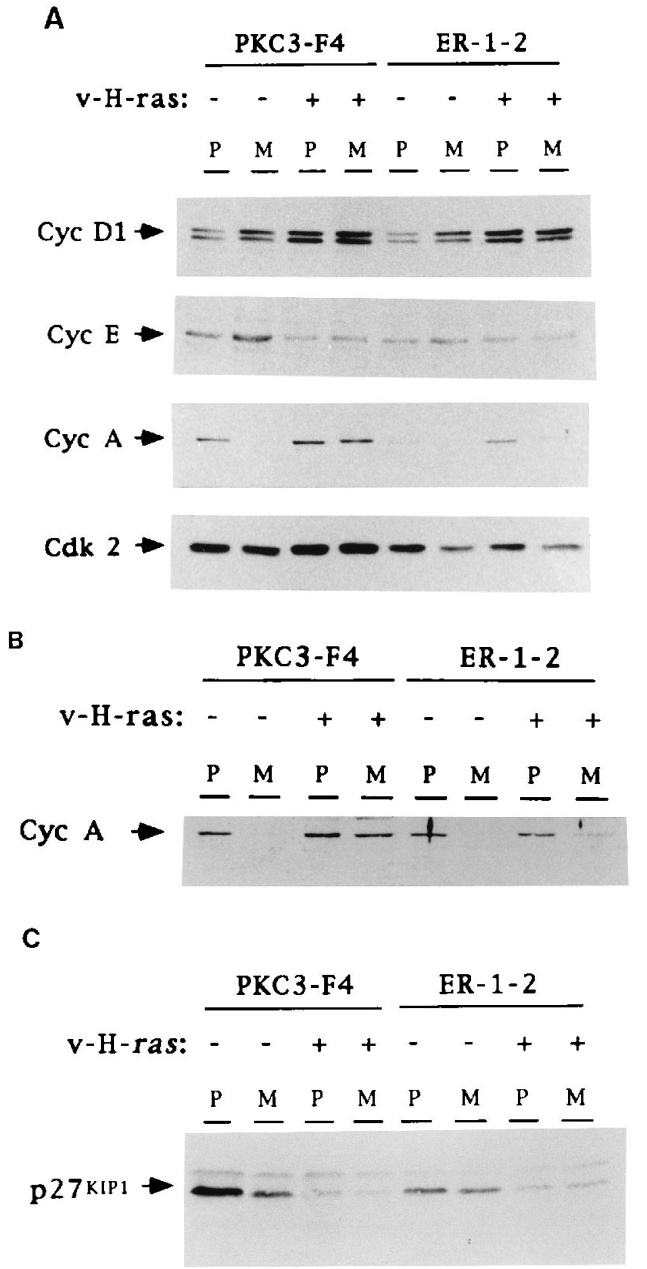

FIG. 3. Expression of various cell cycle-regulatory proteins in PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cells cultured under adherent and nonadherent conditions. Cells grown on plastic cell culture plates (i.e., adherent cultures [P]) or in 1.3% methylcellulose cultures (i.e., nonadherent cultures [M]) were lysed, and 50 μg of total-cell extract was analyzed by Western blotting and probing with specific antibodies, as described in Materials and Methods. (A) Expression of cyclins D1, E, and A and Cdk2. Cyclin D1 often appears as a double band in Western blots made with extracts from rodent cells (65). (B) Expression of cyclin A in adherent and nonadherent cultures of the indicated type that had been arrested at the G_1/S-phase border. See the text for details. (C) Expression of $p27^{Kip1}$ in the indicated cell types. + and −, expressing or not expressing v-H-*ras*, respectively.

might proceed without further need for adhesion. To begin to address this question, we analyzed the effects of adhesion and *ras* in PKC3-F4 and ER-1-2 cells on two cell cycle events that occur prior to cyclin A expression and that are critical for the G_1-to-S phase transition: phosphorylation of the product of the retinoblastoma tumor suppressor gene, pRB (reviewed in reference 71), and cyclin E-dependent kinase activity (46, 47, 54). Adherent and nonadherent cultures of PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cells were prepared as described above for analysis of cyclin expression. Western blots of extracts derived from such cultures were probed with an antibody to pRB, and the hypo- and hyperphosphorylated forms of pRB were distinguished by their respective mobilities on polyacrylamide gels (38). Figure 5 demonstrates that both forms of pRB were detectable in adherent cultures of PKC3-F4 and ER-1-2 cells but that only the hypophosphorylated form was present in nonadherent cultures of these two cell lines. PKC3-F4/*ras* cells, on the other hand, exhibited mainly hyperphosphorylated pRB when grown in adherent or nonadherent methylcellulose cultures. In contrast to what was observed with cyclin A expression, the hyperphosphorylated form of pRB was very abundant in ER-1-2/*ras* cells in both adherent and nonadherent cultures.

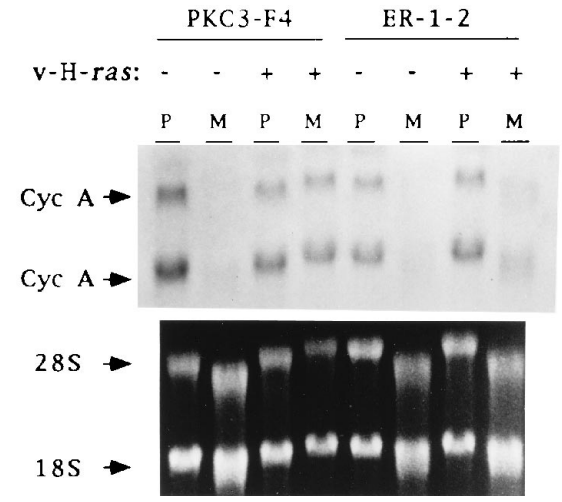

FIG. 4. Northern blot analysis of expression of cyclin A mRNA in PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cell lines cultured under adherent and nonadherent conditions. Cells grown on plastic cell culture plates (i.e., adherent cultures [P]) or in 1.3% methylcellulose cultures (i.e., nonadherent cultures [M]) were lysed, and 9 μg of total cellular RNA from each cell type was analyzed by Northern blotting and hybridization to a ^{32}P-labeled human cyclin A probe (35). The 3.0- and 1.8-kb cyclin A bands are indicated, and the ethidium bromide-stained gel, featuring the 28 and 18S rRNA bands, is shown as a loading control. The variation in electrophoretic mobility is due to the presence in some samples of methylcellulose-derived insoluble material that is difficult to remove during isolation of RNA from cells cultured in such medium. + and −, expressing or not expressing v-H-*ras*, respectively.

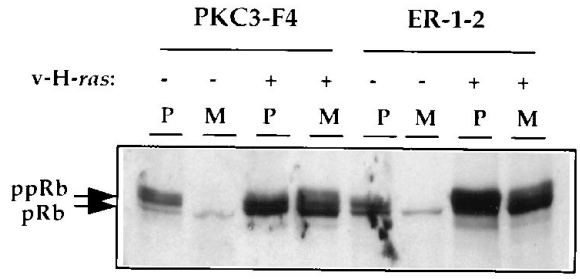

FIG. 5. Western blot analysis of expression and phosphorylation state of pRB in PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cell lines cultured under adherent (P) and nonadherent (M) conditions. Fifty micrograms of total cellular protein was fractionated on an SDS–7.5% polyacrylamide gel and blotted to a nitrocellulose membrane. The blot was then probed with a specific anti-pRB monoclonal antibody. See Materials and Methods for additional details. + and −, expressing or not expressing v-H-*ras*, respectively.

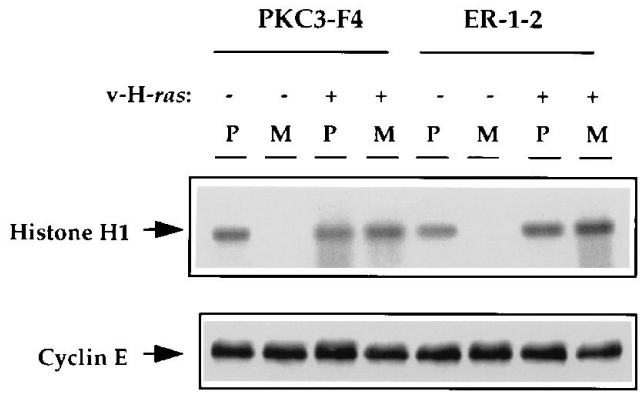

FIG. 6. Cyclin E-dependent kinase activity in PKC3-F4, PKC3-F4/*ras*, ER-1-2, and ER-1-2/*ras* cell lines cultured under adherent (P) and nonadherent (M) conditions. Cell extracts (500 μg) were immunoprecipitated with anti-cyclin E antibodies and analyzed for associated histone H1 kinase activity as described in Materials and Methods. The bottom panel shows a Western blot of aliquots of immunoprecipitates that had been probed with anti-cyclin E antibody, demonstrating that approximately equivalent levels of cyclin E protein were associated with each immunoprecipitate. + and −, expressing or not expressing v-H-*ras*, respectively.

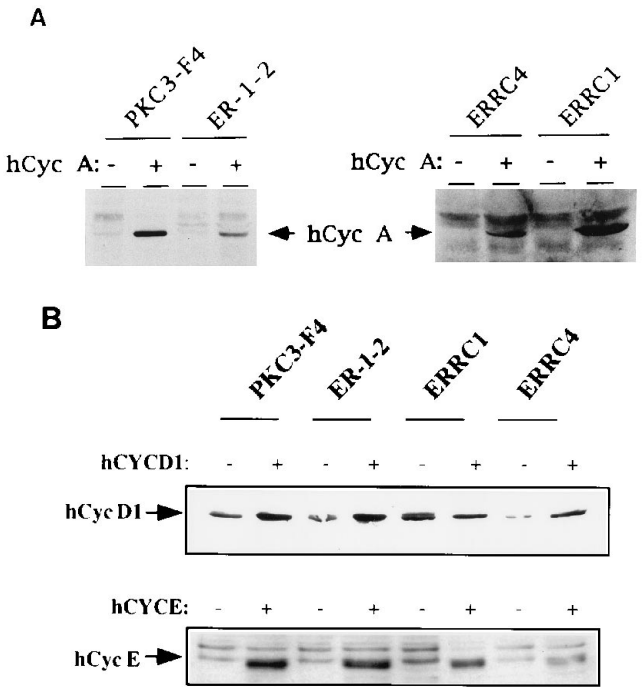

FIG. 7. Analysis of ectopic expression of cyclins A, D1, and E in the PKC3-F4, ER-1-2, ERRC1, and ERRC4 cell lines. Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies specific for human cyclin A (PharMingen), D1 (UBI), or cyclin E (UBI). (A) Ectopic expression of cyclin A. Cells were infected with pBabePuro vector that lacked a cDNA insert (−) or with pBabePuro vector that harbored a human cyclin A (hCyC A) cDNA (+). (B) Ectopic expression of cyclins D1 and E. Cells were infected with pBabePuro vector that lacked a cDNA insert or with pBabePuro vector that harbored human cyclin D1 or human cyclin E cDNA (hCyCD1 and hCyCE, respectively).

Thus, pRB phosphorylation, like cyclin A expression, is an adhesion-dependent cell cycle event that occurs in the absence of adhesion when cells express a *ras* oncogene. In contrast to the case for cyclin A expression, however, ER-1-2/*ras* cells were able to carry out anchorage-independent phosphorylation of pRB, despite their inability to proliferate under such conditions.

Phosphorylation of pRB in mid- to late G_1 phase is thought to be performed largely by cyclin D-dependent kinases (i.e., Cdk4 and Cdk6 [14, 31, 44]), and pRB may be the single critical substrate of these kinases for regulation of cell cycle progression (39, 40). Cyclin E-dependent activation of Cdk2 may also participate in phosphorylation of pRB (25), but cellular substrates for cyclin E-mediated phosphorylation are not known with certainty. Furthermore, cyclin E-dependent events other than pRB phosphorylation are required for the G_1-to-S transition (47, 56). We therefore analyzed cyclin E-dependent kinase activity by immunoprecipitating cyclin E from cells that were cultured as described above and then performing in vitro kinase assays with histone H1 as a substrate. Cyclin E-dependent histone H1 kinase activity was almost exclusively adhesion dependent in PKC3-F4 and ER-1-2 cells, but this adhesion requirement was abrogated in both PKC3-F4/*ras* and ER-1-2/*ras* cells (Fig. 6). Thus, similar to pRB phosphorylation but in contrast to cyclin A expression, the adhesion requirement for mounting a key cell cycle event was lost in ER-1-2/*ras* cells, despite the inability of these cells to proliferate in an anchorage-independent manner. Taken together, these data suggest that adhesion may regulate multiple signaling pathways that impinge on several important cell cycle events. Furthermore, the adhesion dependence of pRB phosphorylation and cyclin E-dependent kinase activity could be dissociated from the adhesion dependence of cyclin A expression, in that the former two events were overcome by expression of *ras* in ER-1-2 cells while the latter was not.

Ectopic expression of cyclin A rescues anchorage-independent growth of ER-1-2/*ras* cells. The data described above indicated that when ER-1-2/*ras* cells were deprived of adhesion, these cells arrested in late G_1 and S phases with hyperphosphorylated pRB and cyclin E-dependent kinase activity but also with very low levels of cyclin A. To test whether reduced levels of cyclin A were the only obstacle to anchorage-independent growth of ER-1-2/*ras* cells, we sought to restore expression of this protein in these cells. Two independent subclones of ER-1-2/*ras* cells (75) (designated ERRC1 and ERRC4 in this study), as well as PKC3-F4 and ER-1-2 cells, were infected with a recombinant retrovirus that drives expression of a human cyclin A cDNA from the viral long terminal repeat. The parental vector, pBabePuro (45), also confers resistance to the drug puromycin. The cells were infected with either the cyclin A-expressing virus or, as a control, a virus that lacked a cDNA insert. The infectants were selected in puromycin-containing medium; puromycin-resistant colonies were then pooled and assayed for expression of human cyclin A and colony formation in soft agar. Constitutive expression of cyclin A has been difficult to achieve in many cell types (21, 57), and the number of puromycin-resistant colonies obtained from cyclin A virus infectants was significantly lower than the number obtained with several other genes (29). Nevertheless, at least 100 puromycin-resistant colonies were pooled for each cell type studied. A similar number of control-vector-infected, puromycin-resistant colonies were pooled for comparison. Analysis of ectopic expression of cyclin A was performed by probing Western blots of whole-cell extracts with an antibody specific for human cyclin A. As can be seen in Fig. 7A, all the cyclin A virus infectants, but not the control virus infectants, produced an immunoreactive product of ~58 kDa. It should be noted that the ER-1-2/*ras* subclone, ERRC1, produced larger quantities of cyclin A than did the other subclone, ERRC4. We then tested the ability of these infectants to form colonies in soft

TABLE 1. Soft-agar colony formation by various cell lines infected with various cyclin-expressing retroviruses

Cell line	Growth in soft agar (no. of colonies/10^4 cells)[a]			
	+ pBabePuro	+ Cyclin D1	+ Cyclin E	+ Cyclin A
PKC3-F4	0	0	0	0
ER-1-2	0	0	0	0
ERRC1[b]	0[c]	0	0	384
ERRC4[b]	0	0	0	1,050

[a] A total of 10,000 cells of each cell line were seeded into 0.3% agar, and macroscopic colonies were scored after 2 weeks of growth. + pBabePuro, cells infected with a retroviral vector lacking a cDNA insert; + cyclin D1, E, or A, cells infected with a retroviral vector that drives expression of a human cyclin cDNA of the indicated type.

[b] ERRC1 and ERRC4 are two independent clonal cell lines derived from ER-1-2/*ras* cells (75).

[c] In most experiments, the ERRC1 cells infected with the pBabePuro vector formed no colonies; however, in some experiments, these cells formed small numbers of colonies, never exceeding $51/10^4$ cells.

agar. Table 1 and Fig. 8 demonstrate that infection with the cyclin A-expressing virus, but not the virus lacking an insert, rescued anchorage-independent growth of both ERRC1 and ERRC4 cells. Ectopic expression of cyclin A was not sufficient,

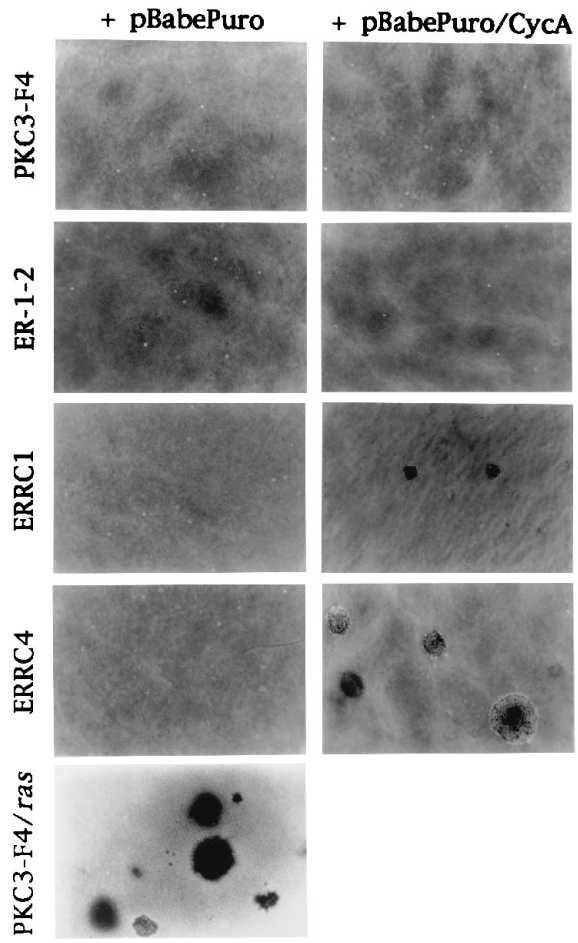

FIG. 8. Growth of PKC3-F4, ER-1-2, ERRC1, and ERRC4 cells in soft agar in response to ectopic expression of cyclin A. The cells were infected with control or cyclin A-expressing retroviral vectors as described in the text. Medium containing 0.3% soft agar was inoculated with 10^4 cells of each type; macroscopic colonies were scored after 2 weeks of growth. PKC3-F4/*ras* cells are shown as a positive control. See Table 1 for quantitation of results.

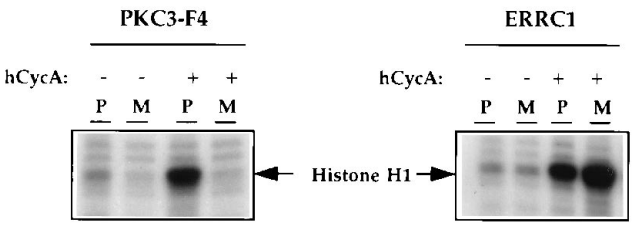

FIG. 9. Cyclin A-dependent kinase activity in PKC3-F4 and ERRC1 cell lines that ectopically express human cyclin A when cultured under adherent (P) and nonadherent (M) conditions. Cell extracts (500 μg) were immunoprecipitated with anti-human cyclin A antibodies and analyzed for associated histone H1 kinase activity as described in Materials and Methods. A Western blot of aliquots of the immunoprecipitates were probed with anti-human cyclin A antibody to demonstrate that approximately equivalent levels of cyclin A protein were associated with immunoprecipitates from adherent and nonadherent cultures of PKC3-F4 and ERRC1 cells infected with the cyclin A-expressing retrovirus (data not shown). hCycA, human cyclin A.

however, to induce anchorage-independent growth of either PKC3-F4 or ER-1-2 cells under these conditions (i.e., agar-containing medium supplemented with 5% calf serum). PKC3-F4/*ras* cells form colonies in soft agar under these conditions at an efficiency of 10 to 20% (75). Thus, the ERRC4 cell line was rescued to a nearly wild-type frequency of colony formation by ectopic expression of cyclin A. In contrast, over the course of several experiments, the cyclin A-expressing ERRC1 cells did not grow as well in soft agar, despite producing larger quantities of this protein (Fig. 7). Many of the soft-agar colonies that were formed by cyclin A-expressing ERRC1 cells died after 5 to 7 days of growth and lysed in the agar medium. Cyclin A must be destroyed for cells to exit M phase (59); we suspect that the larger quantity of constitutively expressed cyclin A that these cells produced was, for unknown reasons, particularly toxic when these cells were cultured in the absence of adhesion.

Because ectopic expression of cyclin A did not induce anchorage-independent growth of PKC3-F4 or ER-1-2 cells, and pRB phosphorylation and cyclin E-dependent kinase activity were both adhesion dependent in these cell lines, we next analyzed histone H1 kinase activity associated with human cyclin A in infected PKC3-F4 and ERRC1 cells that had been cultured under adherent or nonadherent conditions. As shown in Fig. 9, PKC3-F4 cells infected with the cyclin A-expressing virus that were cultured under adherent conditions exhibited significantly increased kinase activity in cyclin A immunoprecipitates, relative to that of control-virus-infected cells. This enhanced activity was not observed, however, when the PKC3-F4 cells were cultured under nonadherent conditions. In contrast, enhanced cyclin A-associated kinase activity was observed in ERRC1 cells regardless of their adhesive state. Thus, as seen with pRB phosphorylation and cyclin E-dependent kinase activity (Fig. 5 and 6), kinase activity associated with ectopically expressed cyclin A was dependent on cell adhesion, and this dependence was overcome by expression of oncogenic *ras*. These data are consistent with the notion that the adhesion dependence of the activity of certain Cdks can be dissociated from the adhesion dependence of cyclin A expression.

Finally, to test the specificity of the cyclin A-mediated rescue of anchorage-independent growth of ERRC1 and ERRC4 cells, human cyclins D1 and E were also ectopically expressed in these and the control cell lines. Ectopic expression of these cyclins was assessed by probing Western blots with antibodies that recognized both endogenous (rat) and exogenous (human) cyclin D1 or only the exogenous human cyclin E (Fig.

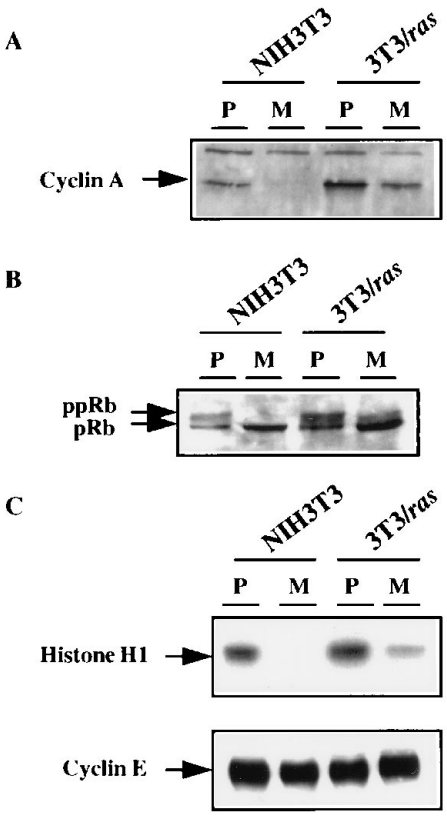

FIG. 10. Effects of adhesion and *ras* on cell cycle events in NIH 3T3 cells. (A) Western blot analysis of cyclin A expression. (B) Western blot analysis of pRB phosphorylation. ppRb, phosphorylated pRB. (C) Cyclin E-dependent histone H1 kinase activity; the bottom panel shows a Western blot of aliquots of immunoprecipitates that had been probed with anti-cyclin E antibody, demonstrating that approximately equivalent levels of cyclin E protein were associated with each immunoprecipitate. P, adherent cultures; M; nonadherent methylcellulose cultures. Western blot analyses and kinase assays were performed as described in Materials and Methods and in the legends to Fig. 3, 5, and 6.

7B). If the antibody to cyclin D1 recognized rat and human forms equally well, only about a twofold enhancement of cyclin D1 protein expression was achieved in these cell lines, perhaps because of the relatively high levels they produce endogenously (Fig. 2A). In contrast to the results with cyclin A, ectopic expression of either cyclin D1 or cyclin E failed to rescue the ability of ERRC1 or ERRC4 cells to form colonies in soft agar (Table 1). Thus, overproduction of other G_1 cyclins could not substitute for cyclin A in mediating *ras*-induced, anchorage-independent growth.

Effects of adhesion and *ras* on pRB phosphorylation, cyclin E-dependent kinase activity, and cyclin A expression in NIH 3T3 cells. To confirm and extend the data obtained with our rat 6-derived cell system, we analyzed the effects of cell adhesion and expression of oncogenic *ras* on cyclin A expression, pRB phosphorylation, and cyclin E-dependent kinase activity in another rodent fibroblast cell line, NIH 3T3. Parental, anchorage-dependent NIH 3T3 cells and NIH 3T3 cells transformed by a human K-*ras* oncogene (3T3/*ras* cells) were cultured under adherent and nonadherent conditions as described above. As can be seen in Fig. 10A, expression of cyclin A was adhesion dependent in the parental NIH 3T3 cells, but this adhesion requirement was abrogated in the 3T3/*ras* cells. Also similar to the rat 6-derived cell lines, phosphorylation of pRB was anchorage dependent in NIH 3T3 cells (Fig. 10B). Furthermore, the hyperphosphorylated form of pRB was observed in nonadherent, methylcellulose cultures of 3T3/*ras* cells, although the extent of hyperphosphorylation in such cultures was not as great as that observed for PKC3-F4/*ras* or ER-1-2/*ras* cells (Fig. 5). Finally, cyclin E-dependent kinase activity was also adhesion dependent in the parental cell line, but this adhesion dependence was overcome when the cells expressed oncogenic *ras* (Fig. 10C). We conclude that the three events defined as adhesion dependent in the rat 6-derived cell lines were also adhesion dependent in NIH 3T3 cells; furthermore, expression of a *ras* oncogene supplanted the adhesion requirement of these events in both cell types.

DISCUSSION

Anchorage-independent proliferation is a hallmark of transformed cells, but little is known of the mechanism(s) by which oncogenes induce this phenomenon. We have developed a system that allows analysis of oncogene-induced, anchorage-independent growth in isolation from many other transformation-associated phenotypes. The ER-1-2 variant cell line responds to expression of the v-H-*ras* oncogene with alterations in cell morphology and gene expression that are indistinguishable from those of a control cell line, PKC3-F4 (16, 32). In contrast to PKC3-F4 cells, however, ER-1-2 cells fail to form colonies in soft agar in response to *ras* (32).

We report here that three critical cell cycle events were dependent on adhesion to a substratum in PKC3-F4 and ER-1-2 cells: pRB phosphorylation, cyclin E-dependent kinase activity, and expression of cyclin A. Certain other cell cycle events, such as expression of cyclins D1 and E, were not regulated by adhesion in these cells. These data offer at least a partial molecular explanation for the adhesion requirement of cells to proliferate; i.e., adhesion-mediated signals and/or adhesion-associated alterations in cellular architecture (9, 74) are necessary for appropriate activation of the cell cycle machinery. Strikingly, all three of these cell cycle events occurred in the absence of adhesion following expression of *ras* in PKC3-F4 cells. Thus, an oncogene can usurp cellular functions required for cell cycle progression that are normally regulated by adhesion. These data therefore also offer a plausible mechanism for oncogene-induced, anchorage-independent growth. Importantly, very similar data were obtained for NIH 3T3 cells.

Surprisingly, it was found that ER-1-2/*ras* cells displayed hyperphosphorylated pRB and cyclin E-dependent kinase activity in the absence of adhesion, despite their inability to proliferate in an anchorage-independent fashion. In this regard, these cells resembled PKC3-F4/*ras* cells, which could proliferate in semisolid media. In contrast to the situation in PKC3-F4/*ras* cells, however, expression of cyclin A in ER-1-2/*ras* cells remained dependent on adhesion. Thus, the adhesion dependence of pRB phosphorylation and cyclin E-dependent kinase activity could be dissociated from the adhesion dependence of cyclin A expression, in that the dependence of the first two events was overcome in the ER-1-2 cells by expression of *ras*, while in the last it was not. Furthermore, ectopic expression of cyclin A in PKC3-F4 cells led to enhanced histone H1 kinase activity only when these cells were adherent. In contrast, ectopic production of cyclin A in *ras*-expressing ER-1-2 cells led to enhanced histone H1 kinase activity regardless of the adhesive state of the cells. This observation, along with the adhesion dependence of pRB phosphorylation and cyclin E-associated kinase activity, suggests that adhesion could be a general requirement for activation of G_1 Cdks, at least in rat 6 cell lines. It is possible, therefore, that adhesion may regulate

multiple and/or branching signal transduction pathways in rat 6 cells and that distinct pathways may control (i) pRB phosphorylation and cyclin E and A-dependent kinase activity (i.e., G_1 Cdk activation) and (ii) expression of cyclin A. This is not surprising, given the likely role of integrins in the adhesion requirement for cell proliferation and the complexity of integrin-mediated signal transduction (9, 74). The mechanism(s) by which adhesion regulates G_1 Cdk activity in rat 6 cells is unclear, but preliminary investigations of this question indicate that the levels of Cdk2 and p27 that coimmunoprecipitate with cyclin E are unaltered by cell adhesion (29).

Subsequent to submission of this paper, Fang et al. reported the adhesion dependence of cyclin E-Cdk2 activity in human fibroblast cell lines (15). The lack of such activity appeared to result from increased expression of the Cdk2 inhibitors p27 and p21 in suspended cells. This is unlikely to be the mechanism of the adhesion dependence of cyclin E-associated kinase activity in PKC3-F4 and ER-1-2 cells, since p27 levels were not altered by the adhesive state of these cells, nor did the cells produce detectable levels of p21. Consistent with this difference, pRB phosphorylation occurred in the absence of adhesion in the human cell lines (15), whereas it failed to occur in nonadherent cultures of the rodent cell lines used in this study. These disparities are more likely to represent cell type differences than species differences because Bohmer et al. have also very recently reported that pRB phosphorylation is adhesion dependent in early-passage human foreskin fibroblast cultures (7). It should be noted that the lack of pRB phosphorylation in those cells was linked to adhesion dependence of cyclin D1 expression (7). In contrast, the rat 6 cell lines described here produced cyclin D1 in nonadherent cultures and still failed to phosphorylate pRB. Apparently, adhesion-mediated regulation of the cell cycle machinery is very complex, with several different mechanisms being operative in several different cell types. Fang et al. also suggested that the absence of cyclin A in NRK cells deprived of adhesion (21) is likely to be a consequence of cell cycle arrest in late G_1 phase due to a lack of cyclin E-Cdk2 activity, rather than the cause of the arrest (15). The data reported here suggest an alternative possibility, namely, that cyclin E-Cdk2 activity and cyclin A expression each require an independent adhesion-mediated signal.

Phosphorylation of pRB (presumably mediated by cyclin D-Cdk4 and Cdk6 activity [14, 31, 44]), induction of cyclin E-Cdk2 activity, and expression of cyclin A are each required for progression through late G_1 phase and entry into S phase (26, 27, 63, 71). The failure of PKC3-F4 and ER-1-2 cells to perform any of these three important cell cycle events when cultured under nonadherent conditions is consistent with the very tight arrest of these two cell lines in G_1 phase that was also observed under such conditions. It has been proposed that cyclins D, E, and A act sequentially to orchestrate the G_1-to-S phase transition (47). Cyclin A may also play a role in regulation of S phase itself (26). Of the cell cycle events analyzed in this study, only cyclin A expression was dependent on adhesion in ER-1-2/*ras* cells, with only trace amounts of this protein produced when these cells were cultured in methylcellulose-containing medium. Two lines of evidence indicate that this is the major, if not the only, obstacle to anchorage-independent growth of these cells. First, ER-1-2/*ras* cells arrested later in the cell cycle than PKC3-F4 or ER-1-2 cells when cultured under nonadherent conditions, i.e., in both late G_1 and S phases. Second, ectopic expression of cyclin A, but not of cyclin D1 or E, was sufficient to rescue anchorage-independent growth of ER-1-2/*ras* cells. It appears, therefore, that the putative dominant mutation in the ER-1-2 cell line (32) acts quite specifically to block the ability of *ras* to activate an adhesion-regulated pathway that controls expression of cyclin A. We conclude that the ability of *ras* to direct cyclin A expression in the absence of cell-substratum adhesion may be critical to the induction of anchorage-independent growth by this oncogene. A similar conclusion has recently been drawn for anchorage-independent growth of rat 1a cells that express the c-*myc* oncogene (4). The signaling pathway(s) utilized by Ras that ultimately leads to anchorage-independent expression of the cyclin A gene is unknown. Prominent among the numerous candidate pathways controlled by Ras is the mitogen-activated protein (MAP) kinase pathway; however, defects in this pathway are unlikely to account for the failure of ER-1-2/*ras* cells to form colonies in soft agar, because we detect no significant differences in MAP kinase activity between PKC3-F4 and ER-1-2 cells or their respective *ras*-expressing derivatives, nor can elevation of MAP kinase activity via expression of a constitutively activated allele of MAP kinase kinase rescue anchorage-independent growth of ER-1-2/*ras* cells (76).

While ectopic expression of cyclin A rescued anchorage-independent growth of ER-1-2/*ras* cells, it was not sufficient to induce anchorage-independent growth of the PKC3-F4, ER-1-2, or parental rat 6 (29) cell line. Therefore, *ras*-mediated functions other than anchorage-independent expression of cyclin A are also likely to be necessary for anchorage-independent cell proliferation. Because the ectopically expressed cyclin A was not associated with significant kinase activity in nonadherent cultures of PKC3-F4 cells but was associated with kinase activity in nonadherent cultures of *ras*-expressing ER-1-2 cells, it is reasonable to speculate that one such function would be to direct anchorage-independent activation of G_1 Cdks, leading to phosphorylation of pRB and other targets. It is worth noting that White et al. have demonstrated that at least two independent Ras functions can contribute to transformation of mammalian cells (72). Perhaps these two functions lead individually to anchorage-independent activation of G_1 Cdks and expression of cyclin A. Experiments aimed at determining the nature of the additional functions required for anchorage-independent growth by rat 6 cells, as well as the *ras*-mediated signaling pathways that usurp adhesion dependence of cell proliferation, are under way.

ACKNOWLEDGMENTS

We thank R. Assoian and J. Roberts for generously providing cyclin A and cyclin E antibodies, respectively, and for communicating results prior to publication. We also thank Danny Lew and Steve Reed for the human cyclin A and cyclin E cDNAs, Wei Jiang and Bernie Weinstein for the human cyclin D1 cDNA, Tom Hei and Scott Kahn for the NIH 3T3 and NIH 3T3/*ras* cell lines, Su-Jae Lee for technical advice, S. Arkin and C. Lackner for assistance with the flow cytometry analyses, and J. Hirsch, J. Manfredi, S. Kohtz, M. Sudol, and G. Acs for comments on the manuscript.

This work was supported by NIH grant CA59474 and by a Sinsheimer Scholar's Award to R.S.K.

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