Lower Cyclin H and Cyclin-dependent Kinase-activating Kinase Activity in Cell Cycle Arrest Induced by Lack of Adhesion to Substratum

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ABSTRACT
Knowledge about adhesion checkpoints is important to counteract dissemination of cells from solid tumors. Lack of anchorage in adherent cells is associated with growth arrest and inhibition of cyclin-dependent kinases (cdks) required to drive cell cycle progression. Because cyclin-cdk complex activation requires CDK-activating kinase comprising cdk7 and cyclin H, we now investigated their relationship to decreased proliferation by lack of cell spreading. This report shows that either UV irradiation on an adhesive substrate or culture on a nonadhesive substrate produced K1735 melanoma growth arrest. Inhibition of proliferation by UV primarily induced the cdk inhibitor p21WAF1 without a significant effect on cyclin H and cdk7. In contrast, lack of adhesion to substratum decreased cyclin H but not cdk7 with accumulation of a slower migrating, presumably unphosphorylated cdk4 isoform. These results were paralleled by decreased cdk7-mediated phosphorylation of GST-cdk2 and lower activation of a baculovirus-derived cdcd2-cyclin B kinase complex. This is the first report showing that cyclin H-mediated down-regulation of cdks-kinase activating kinase activity is involved in growth arrest induced by lack of anchorage.

INTRODUCTION
Although both primary and immortalized adherent cells undergo growth arrest in suspension, the nature of this arrest is markedly different. Primary cells exhibit growth inhibition involving rapid cessation of DNA synthesis. In contrast, immortalized nontumorigenic cells show growth arrest as measured by thymidine incorporation, but cells that have entered S-phase continue into G2-M and accumulate as a 4N population (1). Many cell type-specific differences have been disclosed between not only normal and transformed cells but also between adherent cell lines, the proliferation of which is strictly anchorage dependent. These apparent discrepancies are seen in established cell lines, most probably because of adaptive events that have occurred during in vitro culture (2). Among the molecular mediators of growth arrest by lack of anchorage, cyclin A, which associates with cdc2 and cdk2 4 to drive cell cycle progression into S-phase and mitosis, is not expressed in suspended primary keratinocytes (2). A similar cyclin A alteration is found in unanchored NIH3T3 cells, but overexpression of cyclin D1 restores expression of cyclin A in these cells and rescues them from cell cycle arrest (3). Also, the cyclin E-cdk2 complex, which is required for the G1-S transition of the cell cycle, is activated in the late G1 phase in attached human fibroblasts but not in fibroblasts maintained in suspension, except in transformed fibroblasts in which the complex is active regardless of anchorage. The lack of cyclin E-cdk2 activity in suspended normal cells was reported to result from increased expression of the p21Cip1 (p21) and p27Kip1 (p27) CDK2 inhibitors (4). Also, cytoplasmic sequestration of these cdk inhibitors away from the nucleus by cytoplasmic cyclin-cdk complexes is also seen in anchorage-independent cells (5). Others have also shown that nonadherent cells fail to phosphorylate the retinoblastoma protein (Rb), but enforced expression of cyclin D1 rescues Rb phosphorylation and entry into S-phase when G1 cells are cultured in the absence of substratum. Nonadherent cells also fail to activate the cyclin E-associated kinase, and this effect can be linked to an increased association of the cdk inhibitors, p21 and p27 (6).

Additionally, up-regulation of p27 Kip1 has been reported to correlate inversely with anchorage-independent growth of human cancer cell lines (7). Another effect associated with anchorage-independent inhibition of growth is a concomitant decrease in phosphorylation of CDK2 on threonine 160 (4). Also, Evi-transformed fibroblasts are anchorage independent, have an abbreviated G1 phase of the cell cycle, and a reduced requirement for serum mitogens for S-phase entry. Evi-mediated transformation is paralleled by elevated cyclin A-cdk2 activity, associated with a combination of increased accumulation and stabilization of cyclin A bound to a faster-migrating species of cdk2 believed to be the active threonine 160-phosphorylated form (7). This phosphorylation of cdk2 and cdk4 is dependent on CAKs, the activity of which depends on the association of cdk7 and cyclin H (8). In human diploid fibroblasts, phorbol esters added within 5 h of serum stimulation inhibited G1-S cell cycle progression and cyclin E-cdk2 activity indirectly by inhibiting message levels for both cdk7 and cyclin H (8).

More recently, a TGF-β1-mediated G1 arrest of HepG2 cells was reported to involve down-regulation of a 45,000 CAK activity, known to activate Cdk2 also via Thr-160 phosphorylation (10). Therefore, we now investigated whether growth inhibition by lack of anchorage of susceptible cells also interferes with cdk activity by modulating CAK activation (9, 10). This was now investigated not only by measuring substrate-dependent changes in CAK kinase activity (9, 10) but also by determining differential protein expression in cyclin H or cdk7 induced by lack of anchorage in K1735 melanoma cells.

MATERIALS AND METHODS
Cell Culture. K1735 nonmetastatic melanoma cells were cultured in DMEM supplemented with 10% serum (11). For studies involving growth arrest by lack of anchorage, cells were seeded on polystyrene bacterial plates (V W R # 25384) coated with 10 mg/ml BSA in PBS (pH 7.2) to prevent cell attachment to substratum (12).

Total Cell Lysates. Cells were collected by scraping with a rubber policeman after washing in ice-cold PBS (pH 7.2) containing 0.1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride. Cells were kept at −70°C until lysis, which was carried out in 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM N-tosyl-L-phenylalanine chloromethyl ketone, and 0.4% NP-40.

Immunoblotting. Total cell lysates (100 μg) were run on 10–12% SDS-polyacrylamide gels concurrently with prestained molecular weight markers (Amersham) and were transferred passively to parallel replicate nitrocellulose
membranes (11). Blotted membranes were blocked with 5% skimmed milk in Tris-buffered saline for 2 h at room temperature and then incubated for 1 h in the same blocking solution containing the specific antibodies. The antibodies used for specific immune blotting of cyclin D3 (SC 182), p21WAF1 (SC-397), cyclin H (SC 609), and cdk7 (SC 529) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immune detection of cdk2, cdk4, and cyclin D1 was achieved with specific antibodies provided by Dr. M. Pagano (Mitotix, Boston, MA and New York University, New York, NY). This was followed by exhaustive washing in Tris-buffered saline containing 0.1% Tween 20, subsequent incubation with protein A-peroxidase (Amersham) at 1:2000, and development by ECL chemiluminescence (Amersham) and autoradiography. Whenever indicated, the nitrocellulose membranes were stripped at 70°C for 30 min in 62 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol, followed by extensive washing with Tris-buffered saline plus 0.2% Tween 20. Stripped blots were blocked again and reacted with new antibodies and further reaction with peroxidase conjugates and detection by ECL (Amersham). Specific molecular weights for immune blotted bands were established by comparison with coelectrophoresed Rainbow colored markers (Amersham).

CAK Activities. CAK activities were assayed either by directly measuring the phosphorylation of human GST-cdk2 (SC-4069; Santa Cruz Biotechnology) by immune precipitates of cdk7 (SC 529; Santa Cruz Biotechnology) on protein A-beads containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 µCi of [γ-32P]ATP in 10 mM ATP, and 1 mM DTT or indirectly by measuring the ability of equal protein concentration of cell extracts to activate human recombinant cdc2 kinase (Promega V-2891) purified from insect cells infected with a baculovirus vector carrying the genes for human p34cdc2 and cyclin B. The latter assays measured the ability of cell extracts to activate the phosphorylation of histone H1 (10 µg) by 5 units of the baculovirus-derived cdc2-cyclin B complex in kinase buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM MnCl₂, 5 µCi [γ-32P]ATP in 10 µM ATP, and 1 mM DTT.

Histone H1 and Rb Kinase Assays. Histone H1 kinase assays after specific immune precipitation with protein A/G agarose beads and either rabbit antibody to cyclin D3 (SC 182) or mouse antibody to cyclin D1 (SC-8396) were carried out as described previously (13). For Rb kinase assays, histone H1 was substituted by 1 μg of pRb (SC-4112; Santa Cruz Biotechnology), and similar conditions were used for phosphorylation (13).

Densitometric Analysis. After chemiluminescence, data from Western blots were acquired with a Hewlett-Packard Scan Jet IV scanner for quantitation of specific bands with the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

RESULTS

Unequal Regulation of p21WAF1 and D Cyclins in Growth Arrest Induced by UV Radiation or Lack of Anchorage. Preliminary experiments showed that culturing K1735 cells on BSA-coated membranes (11). Blotted membranes were blocked with 5% skimmed milk in Tris-buffered saline for 2 h at room temperature and then incubated for 1 h in the same blocking solution containing the specific antibodies. The antibodies used for specific immune blotting of cyclin D3 (SC 182), p21WAF1 (SC-397), cyclin H (SC 609), and cdk7 (SC 529) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immune detection of cdk2, cdk4, and cyclin D1 was achieved with specific antibodies provided by Dr. M. Pagano (Mitotix, Boston, MA and New York University, New York, NY). This was followed by exhaustive washing in Tris-buffered saline containing 0.1% Tween 20, subsequent incubation with protein A-peroxidase (Amersham) at 1:2000, and development by ECL chemiluminescence (Amersham) and autoradiography. Whenever indicated, the nitrocellulose membranes were stripped at 70°C for 30 min in 62 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol, followed by extensive washing with Tris-buffered saline plus 0.2% Tween 20. Stripped blots were blocked again and reacted with new antibodies and further reaction with peroxidase conjugates and detection by ECL (Amersham). Specific molecular weights for immune blotted bands were established by comparison with coelectrophoresed Rainbow colored markers (Amersham).

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nonadhesive plates (12) prevented cell spreading and promoted cell aggregation, which mostly continued after 5 h of transferring aggregated cells to an adhesive tissue culture plate. Complete spreading occurred when these K1735 cells were directly cultured on tissue culture plates, even after exposing cells to 10 J/m² of UV radiation (Fig. 1, upper panel). However, UV-irradiated adherent cells and cell aggregates showed a similar degree of growth arrest 2 days after each of these treatments (Fig. 1). A comparison of these unequal growth arresting effects on expression of the p21WAF1 cyclin-dependent kinase inhibitor and D cyclins revealed that UV increased p21WAF1 levels by 3-fold, in agreement with results showing that these cells harbor a wild-type p53 (11) and in agreement with the DNA-damaging effect of UV, which activates p21WAF1 (14). No comparable induction occurred in cells arrested by lack of anchorage, which revealed instead a preferential increase in cyclin D3, which persisted even after a 5-h subculturing of the cell aggregates on tissue culture plates. In contrast, no comparable elevation was observed in cyclin D1, which decreased more rapidly after transferring cell aggregates to tissue culture plates (Fig. 2).

Decrease in the Fast-migrating Cdk4 Isoform in Growth Arrest Induced by Lack of Anchorage. In mammalian cells, two types of cdk2 isoforms may be detected, because these enzymes occur as inactive unphosphorylated or as faster-migrating forms when activated by CAK phosphorylation (9, 10). Hence, we investigated whether growth arrest by either lack of anchorage or UV irradiation (Fig. 1) induced changes in cdk2 or cdk4 isoforms. Bidirectional immune blot (11) revealed an altered electrophoretic migration in cdk4 isoforms only with lack of anchorage. Cell aggregates showed most of its cdk4 as a slower-migrating band, in contrast to a faster-migrating cdk4 detected in attached proliferating or UV-irradiated cells. On the other hand, comparable levels of each cdk4 isoform were detectable within 5 h of reattachment of aggregates to tissue culture plates, implying a correlation of lower mobility cdk4 and adhesion. However, no comparable isoform changes were evident in cdk2.

Fig. 3. Reversible decrease in fast-migrating cdk4 with lack of anchorage and reattachment. Replicate blots obtained from bidirectional transfer were assayed for changes in cdk2 and cdk4. Note only lower cdk4-P in adherent cells, only upper cdk4 in unanchored cells, and comparable levels of both upper and lower cdk4 isoforms by 5 h of reattachment of aggregates to tissue culture plates.

Fig. 4. Lack of anchorage is associated with a decrease in cyclin H. Replicate blots obtained from bidirectional transfer were assayed for changes in cdk7 and cyclin H. Note decline in cyclin H without a comparable change in cdk7, with lack of adherence and its partial increase by 5 h of reattachment of aggregates to tissue culture plates. Results were normalized for protein loadings in each lane.
which rather showed a quantitative increase with lack of adhesion (Fig. 3).

Cyclin H Is Preferentially Decreased in Unanchored Cell Aggregates. Because the cdk4 results suggested that lack of anchorage interfered with cdk4 phosphorylation, which involves the cdk7-cyclin H complex (10), we investigated whether this effect was associated with alterations in any of these components. Whereas cdk7 levels were essentially similar, cyclin H was unaffected by UV irradiation but decreased preferentially in unanchored cell aggregates and partly recovered within 5 h of reseeding cell aggregates on tissue culture plates (Fig. 4).

Unequal CAK and Rb Kinase Activities in Adherent and Unanchored Cells. Because growth arrest by lack of anchorage (Fig. 1) preferentially correlated with a decline in cyclin H (Fig. 4) and cyclin H is known to activate the CAK activity in cdk7 (9, 10), we investigated whether Rb kinase associated with cyclin D1 or cyclin D3 and CAK activity differed in adherent and nonadherent cells. For Rb kinase studies, we measured Rb phosphorylation catalyzed by specific immune precipitation of cyclin D1 or cyclin D3, followed by autoradiography (13). This revealed a much lower cyclin D1-dependent Rb phosphorylation in nonadherent cells but a comparable cyclin D3-dependent Rb kinase in both adherent and nonadherent cells (Fig. 5, Left). A clearer correlation of the cyclin H decline with growth arrest induced by lack of anchorage was observed in CAK activity assays, which showed a decreased cdk7-dependent phosphorylation of GST-cdk2 in nonadherent cells, which also showed a decreased ability to activate a baculovirus derived cdc2-cyclin B complex.

DISCUSSION

Prior studies have demonstrated down-regulation of G1 cyclins such as cyclin D1 (6), cyclin E (4–6), and cyclin A (1–3) in growth arrest induced by lack of anchorage in some cell types. The mechanism for this adhesion-dependent regulation seems to differ in normal and immortalized cells (1) and may involve the cytoplasmic displacement of p21 and p27 cdk inhibitors in anchorage-independent cells (6), which emphasizes the role of cdk inhibitors and G1 cyclins in mediating anchorage-regulated growth arrest (5, 7). To define novel changes in cell cycle-controlling proteins specifically associated with lack of adhesion, we searched for differential alterations induced by either UV-mediated growth arrest in adherent cells or those occurring without UV in unanchored cell aggregates cultured on a nonadhesive substrate (12). UV-mediated growth arrest clearly correlated with induction of the cyclin-dependent inhibitor p21WAF1 (11, 14) in K1735 cells, which harbor a wild-type p53 (11), with no such effect apparent with inhibition by lack of anchorage.

Because cell cycle progression not only requires induction of G1 cyclins (1–6) but also requires CAK mediated by cdk7 and cyclin H (9, 10), we investigated whether any one of these components was involved in adhesion-dependent growth arrest. Previously, others demonstrated that inhibition of cell cycle progression with phorbol esters (9) or TGF-β1 (10) decreases CAK activity, but these reports did not implicate preferential changes in cyclin H. Moreover, G1-arrested, TGF-β1-treated HepG2 carcinoma cells showing lower CAK activity retained continued cyclin D:cdk4/6 activity but failed to increase p21WAF1 (10). We now found that the cdk7 activator, cyclin H, is preferentially decreased when growth arrest is induced in K1735 by lack of anchorage but not by UV radiation, demonstrating partial recovery in cyclin H within 5 h of reseeding unanchored cell aggregates on tissue culture plates. Cyclin H decline in nonadherent cells correlated with decreased CAK activity measured by decreased histone H1 phosphorylation of a baculovirus-derived cdc2-cyclin B complex and lower GST-cdk2 phosphorylation, data compatible with induction of a slow-migrating cdk4, believed to be the unphosphorylated cdk isoform (8, 10) by lack of anchorage. Growth-arrested, unanchored aggregates also showed a preferential decrease in cyclin D1-dependent but not in cyclin D3-dependent Rb kinase activity and higher cyclin D3 levels. This may be attributable to a possible role for cyclin D3 in growth arrest induced by lack of anchorage, because a cyclin D3 kinase but not a cyclin D1-associated kinase remains active in nonadherent keratinocytes (15). Also, cyclin D3 was implicated recently as a critical contributor to the irreversible exit of differentiating myoblasts from the cell cycle (16).

To our knowledge, this is the first report describing cyclin H-associated decrease in CAK activity with growth arrest induced by lack of anchorage. Recently, others reported that IFN-α-induced growth arrest involves higher levels of the p21WAF1 cdk inhibitor and lower CAK activity, but lower cyclin H is not involved because CAK is restored after immune depletion of the cdk inhibitor (17). These results showing this novel involvement of cyclin H in adhesion-dependent checkpoints should be valuable in developing antisense strategies against cyclin H in cells from solid tumors, which can become anchorage independent in secondary growth and metastasis.
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