Mini-Review

Anchorage-dependent Cell Cycle Progression

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The concept of anchorage-dependent growth and the close relationship between anchorage independence and tumorigenicity were first appreciated more than a quarter century ago (9, 16, 23, 24). Penman and his coworkers then showed that incubation of cells in the absence of substratum (e.g., tissue culture plastic or purified extracellular matrix protein [ECM]) resulted in an inhibition of mRNA production and protein synthesis (2). These effects became less pronounced with increasing degrees of cell transformation (27). The Folkman laboratory showed that a spread cell shape, rather than adhesion per se, was required for the proliferation of anchorage-dependent cells (8). Like the effects of growth factors, the growth regulatory effects of cell anchorage and cell shape mapped to the G1 phase of the cell cycle. With the recent explosion of information about cell cycle control in general, and cyclin-dependent kinases (cdks)[1] in particular, we have begun to develop a molecular picture that can now explain these fundamental tenets of mammalian cell biology.

Regulation of G1 Phase and DNA Synthesis by the Cyclin-dependent Kinases

Several timely reviews have been written on the cdks, so it is sufficient to say that a subset of these enzymes mediates cell cycle progression through G1 phase. In mammalian cells, the key G1 phase cdks are cdk4 (and its functional counterpart, cdk6) and cdk2. Cdk4/6 and cdk2 are inactive in the absence of their cyclin partner(s), and they are activated by the binding of D type cyclins (D1, D2, or D3 to cdk4/6) or cyclin E (to cdk2). Cyclin A is induced at or near the G1/S boundary; it binds to cdk2 in S phase and is involved in S phase progression. Microinjection of antisense cDNA or antibodies to cyclin A in G1 phase cells blocks subsequent DNA synthesis (10, 19).

In addition to cyclin binding, the activity of the G1 phase cyclin-cdks is affected by the action of specific cdk-inhibitors (CKIs). There are two CKI families: the cip/kips ($p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$) which bind to cyclin E-cdk2, cyclin A-cdk2, and cyclin D-cdk4/6, and the INK4s (p15, p16, p18, and p19) which bind only to cyclin D-cdk4/6. Early studies indicated that the binding of one p21 molecule to cyclin A-cdk2 was insufficient to inhibit kinase activity but that higher order associations would inhibit the cyclin A–dependent kinase (29). This conclusion was then extrapolated to the other p21 family members, but the recent crystal structure of the cyclin A-cdk2-p27 complex shows that one copy of p27 fully inhibits kinase activity (20). The degree to which single/multiple copies of p21 and p27 are able to inhibit cyclin E-cdk2 and cyclin D-cdk4/6 may therefore require reevaluation. There have been no reports of anchorage effects on the INK4s, so they are not discussed further here.

Mitogenic growth factors promote G1 phase cell cycle progression by stimulating the formation or activation of cyclin D-cdk4/6 and cyclin E-cdk2. These mitogenic effects typically involve increases in cyclin D expression and decreases in CKI expression. The active enzymes then phosphorylate the retinoblastoma protein (pRb) and its family member, p107. Hypophosphorylated pRb and p107 form complexes with members of the E2F family, and the complexes act as transcriptional repressors (21, 26). pRb and p107 phosphorylation results in disruption of pRb/E2F and p107/E2F complexes, allowing for the induction of E2F-dependent genes such as cyclin A (5). The recent studies discussed below confirm the critical role of growth factors in stimulating cyclin D– and E–dependent kinase activities, as well as pRb and p107 phosphorylation, but they also indicate that these effects are not likely to occur if cells are growth factor–stimulated in the absence of a substratum. Indeed, it now seems this coordinated control of the G1 cyclin-cdks by growth factors and the ECM underlies the well-established anchorage requirement for the proliferation of nontransformed cells.

Effects of Cell Anchorage on Cyclin D1

Cyclin D1 is the primary D type cyclin for several anchorage-dependent cell types, and recent studies indicate that the ECM and mitogens are jointly required to induce cyclin D1 expression (3, 30). In both NIH-3T3 cells and human fibroblasts, we found that cyclin D1 mRNA and protein were not induced if quiescent cells were stimulated with mitogens in the absence of substratum. The translation of cyclin D1 from preexisting cyclin D1 mRNA is also

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1. *Abbreviations used in this paper*: cdk, cyclin-dependent kinase; CKI, cdk-inhibitor; pRb, retinoblastoma protein.

blocked when cells are incubated in the absence of substratum (30). Adhesion-dependent cyclin D1 expression is causal for cell cycle progression: forced expression of cyclin D1 in nonadherent 3T3 cells rescues pRb phosphorylation and entry into S phase (22, 30).

Effects of Cell Anchorage on Cyclin E-cdk2 Activity

Cyclin E and cdk2 protein levels are only moderately induced in G1 phase, but cyclin E-cdk2 kinase activity is strongly growth factor–dependent and increases dramatically in late G1 phase. This induction fails to occur if cells are stimulated with mitogens in the absence of substratum, presumably because cell adhesion to ECM decreases steady-state levels of the p21-like CKIs. The expression of p21 is increased (7, 30) and the degradation of p27 is decreased (22) in suspended fibroblasts. In contrast, cell adhesion does not have pronounced effects on (*a*) the levels of cyclin E or cdk2, (*b*) formation of the cyclin E-cdk2 complex, or (*c*) the positive and negative regulatory phosphorylations that modulate cdk2 activity (4, 7, 14, 22, 30).

An important feature of the p21 CKI family is that they bind to cyclin D1-cdk4/6 as well as cyclin E-cdk2. The total p21/p27 pool is normally distributed between these cyclin-cdk complexes in G1 phase cells. This fact would suggest that cyclin E-cdk2 activity might also be regulated indirectly by the adhesion-dependent expression of cyclin D1 and formation of cyclin D1-cdk4/6 complexes. Indeed, Zhu et al. (30) found that cyclin E–dependent kinase activity was strictly adhesion-dependent in NIH-3T3 cells even though adhesion to substratum had only minimal effects on total p21/27 levels in this cell line. Cyclin D1 levels were strongly adhesion–dependent in this system, so the absence of cyclin D1, with the consequent reduction in cyclin D-cdk4/6 complexes, probably allowed for a redistribution of the total p21/27 pool in the suspended cells. Cyclin E-cdk2 would be targeted in this redistribution because G1 phase cells were used and hence lacked cyclin A-cdk2 and cyclin B-cdc2.

Thus, through changes in steady-state levels, redistribution, or both, adhesion of cells to substratum reduces the amounts of p21 and p27 that are available to bind to cyclin E-cdk2, and this effect correlates well with the adhesion requirement for cyclin E-cdk2 activity. However, it is worth noting that two studies (4, 14) have observed an inhibition of cyclin E-cdk2 activity in suspended cells that could not be traced to these cdk inhibitors.

Adhesion-dependent Phosphorylation of the Retinoblastoma Protein and p107

Several recent reports show that pRb phosphorylation is dependent upon cell anchorage as well as growth factors (3, 14, 22, 30). Schulze et al. (22) also showed the same result for p107. In one sense these results are expected because cell anchorage and mitogens jointly regulate the induction of cyclin D–dependent and cyclin E–dependent kinase activities (see above). Yet in another sense, the result strongly emphasizes the point that growth factors and the ECM are partners in cell cycle control, with each providing essential signals that allow for proper induction of the G1 cdks and phosphorylation of their substrates. Because cdk-mediated phosphorylation disrupts Rb/E2F and p107/E2F complexes, the result also suggests that E2F-regulated genes in general will be controlled by cell anchorage to substratum.

Adhesion-dependent Expression of Cyclin A

The induction of cyclin A is strongly dependent on signals from the ECM, and several lines of evidence indicate that a large part of this effect is a consequence of adhesion-dependent pRb/p107 phosphorylation. First, there is a close correlation between the adhesion-dependent phosphorylation of pRb (or p107) and expression of cyclin A (14, 22, 30). Second, forced expression of cyclin D1 in nonadherent NIH-3T3 cells rescues pRb phosphorylation, expression of cyclin A, and entry into S phase (22, 30). Third, the overexpression of cyclin D1 transactivates the cyclin A promoter (21). Fourth, the overexpression of E2F1 induces cyclin A expression and anchorage-independent growth (5, 28).

Three laboratories (13, 21, 31) have identified a small GC-rich motif that mediates repression of the cyclin A promoter, and one of the studies (21) also showed that this motif is actually a variant E2F site that binds to E2F4 and E2F4/p107 specifically. This result is attractive because the only other potential E2F sites in the cyclin A promoter are downstream of the transcription start sites, and they appear to be nonfunctional (15). So the suggestion is that cyclin A expression may be repressed in G0 and G1 phase by a variant E2F site that is occupied by an E2F4/p107 complex (21, 22). Cdk-mediated phosphorylation of p107 in mid-late G1 phase would relieve repression by allowing for the dissociation of the complex and release of E2F4. E2F1 binds to this site weakly (31), suggesting that overexpression of E2F1 may stimulate cyclin A expression by displacing the E2F4/p107 complex or by competing for the site with low affinity. The forced expression of cyclin D1 in suspended cells could rescue cyclin A expression directly (through cdk4/6 mediated phosphorylation of p107) or indirectly by sequestering cdk inhibitors (allowing for anchorage-independent cyclin E-cdk2 activity and cyclin E-cdk2–dependent p107 phosphorylation). The latter mechanism is supported by the finding that transfection of a dominant-negative cdk2 blocks G1/S expression of a cyclin A promoter-luciferase reporter construct (4).

An E2F-independent mechanism also seems to be involved in the adhesion-dependent expression of cyclin A. Nonadherent NRK cells and v-ras–infected ER-1-2 cells express cyclin D1, possess cyclin E-cdk2 kinase activity, and phosphorylate pRb when treated with mitogens in suspension (11, 14). Yet cyclin A expression remains adhesion-dependent and forced expression of cyclin A rescues entry into S phase in the absence of substratum (11, 14). This mechanism remains largely uncharacterized, but may involve c-*myc* (1) and/or a CCAAT-binding protein (15).

A Model for the Coordinate Control of G1 Phase Cyclin-dependent Kinases by Growth Factors and the Substratum

Fig. 1 shows a working model of G1 phase cell cycle progression that emphasizes joint control by growth factors and the substratum. Both stimuli are required for induction of cyclin D1 mRNA, and the substratum is also required for the translation of cyclin D1 mRNA. In the dif-

ferent ways discussed above, growth factors and the ECM also control the steady-state levels of p21 and p27. Coordinated titration of cyclin D1, p21, and p27 levels by growth factors and the ECM allows for proper cyclin/cdk/CKI stoichiometry and G1 phase activation of cyclin D– and E–dependent kinase activities. At least in part, these enzymes control the cell cycle by phosphorylating pRb and p107, thereby allowing for the dissociation of E2F and the induction of E2F-dependent genes including cyclin A. The less well characterized E2F-independent induction of cyclin A is also shown.

This model can also account for the anchorage-dependent phenotype of nontransformed cells. In suspended cells, mitogens are unable to induce the expression of cyclin D1 and the formation of cyclin D1-cdk4/6 complexes. So the p21/p27 pool redistributes and binds to cyclin E-cdk2. This effect is complemented by a reduced degradation of p27 and increased expression of p21; studies in vitro suggest that all three effects can contribute to the inhibition of cyclin E kinase activity in suspended cells. The absence of cyclin D1-cdk4/6 and cyclin E-cdk2 activity precludes phosphorylation of pRb/p107 and thereby inhibits E2F-directed expression of the cyclin A gene.

A few issues still need to be resolved. In some studies, nonadherent cells lack both cyclin D-cdk4 and cyclin E-cdk2 activity (22, 30) while others (7) find that only cyclin E-cdk2 activity is adhesion-dependent. We find that adhesion-dependent cyclin D1 expression is typically the determining factor in adhesion-dependent cyclin D-kinase activity (3, 30), but others find that cyclin D-cdk4/6 activity, but not cyclin D1 expression, is blocked in suspended cells (22).

Links between Integrins, the Cytoskeleton, and Anchorage-dependent Cell Cycle Control

Cell anchorage to substratum reflects the interaction of the ECM with integrins, a family of cell surface receptors comprised of α and β chains that heterodimerize in distinct combinations to confer ligand specificity. A few studies have tried to link specific integrins with specific events in anchorage-dependent cell cycle progression. Symington (25) showed that cdk activity and pRb phosphorylation were stimulated when an α5β1 integrin overexpressing K562-subclone was treated with the peptide GRGDS (a ligand for α5β1 integrin). Meredith et al. (18) microinjected a growth inhibitory form of the β1-integrin subunit (called β1c) into 10T1/2 fibroblasts and found that cell cycle progression was blocked in late G1, near or after the induction of cyclin E-cdk2 activity. Nevertheless, detailed studies of specific integrin effects on distinct cyclin-cdk events have yet to be reported.

Part of the difficulty in addressing integrin function with regard to cell cycle control rests in the fact that ECM/integrin binding leads to organization of the cytoskeleton and cell spreading. Both the initial binding event and the subsequent cell spreading may contribute to anchorage-dependent cell cycle control. For example, Hansen et al. (12) reported that integrin binding was sufficient to induce events characteristic of transit through early G1 phase whereas cell cycle progression from mid-late G1 into S phase required cell spreading. This idea is supported by experiments showing that the anchorage requirement for expression of cyclin D1 mRNA and phosphorylation of pRb (mid-G1 events) requires an organized cytoskeleton (a spread cell shape) rather than adhesion per se (3). Perhaps any ECM/integrin interaction that organizes the cytoskeleton will be sufficient to mediate anchorage-dependent cell cycle progression in mid-late G1 phase.

It is important to emphasize that cell adhesion to ECM is not merely permissive for overall growth factor responsiveness. For example, McNamee et al. (17) showed that PDGF phosphorylates its receptor and stimulates phos-

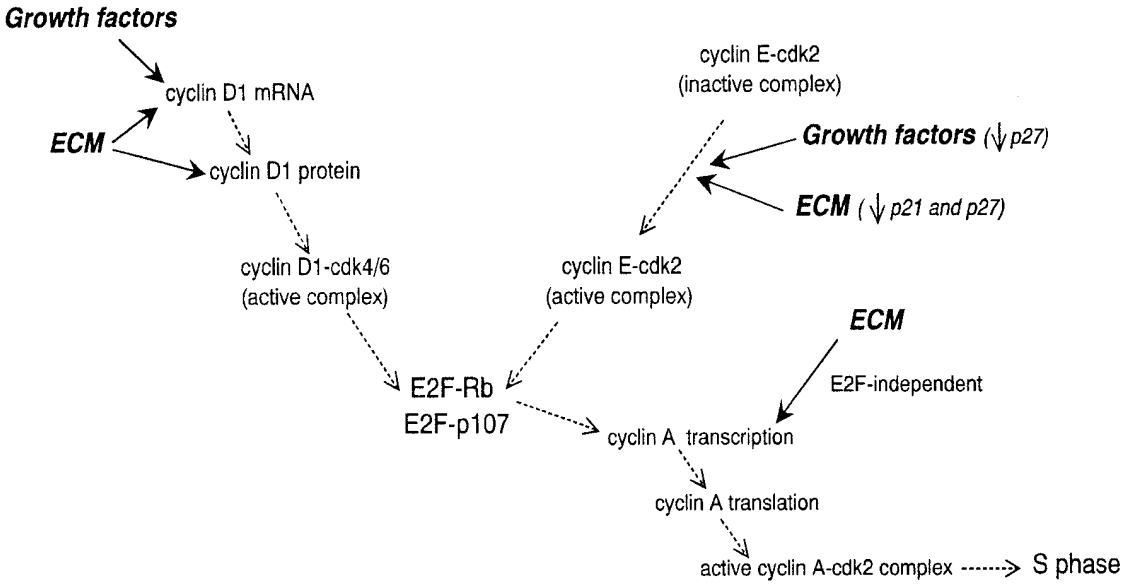

Figure 1. Coordinated control of G1 phase cell cycle progression by growth factors and the extracellular matrix (ECM). A working model showing the major sites of growth factor and ECM action *(solid arrows)* during cell cycle progression from G0 to S phase. Cyclin E gene expression is strongly pRb/E2F dependent, but the cyclin E protein is stable and present in G1 phase cells both before and after pRb phosphorylation. Regulatory phosphorylations and the roles of INK4s are not shown.

pholipase C-γ normally in suspended cells. Moreover, mitogens induce c-*myc* mRNA normally even when cells are cultured in the absence of substratum (3, 6). Although several signaling effects of growth factors and the ECM presently seem redundant (e.g., the induction of MAP kinase and c-*fos*), these individual effects are not sufficient to mediate cell cycle progression into S phase. One of the challenges in this field is to reconcile the apparent redundancy of growth factor and ECM-mediated signal transduction to the fact that growth factors and the ECM have complementary and nonredundant roles in regulating the G1 phase cyclin-cdks.

Concluding Comment

Most of the studies discussed above have been done in fibroblasts. The ECM also controls cell differentiation and survival; these effects are particularly pronounced in epithelial and endothelial cells and preclude using these cell types for many of the kinds of experiments reviewed here. There seem to be important links between cell cycle control, differentiation and survival, and it is not unreasonable to think that these links will also be regulated by signals from the extracellular matrix.

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