Retinoblastoma Susceptibility Gene Product (pRb) and p107 Functionally Separate the Requirements for Serum and Anchorage in the Cell Cycle G1-phase*

Growth factors and cell anchorage are both required for cell cycle G1-phase progression, but it is unclear whether their function is mediated through the same set of cell cycle components and whether they are both required during the same period of time. We separately analyzed the requirements of serum and anchorage during G1-phase progression and found that human dermal fibroblasts as well as wild type, pRb+/−, and p107−/− mouse embryonic fibroblasts needed serum (growth factors) until mid-G1-phase but required cell anchorage until late G1-phase to be competent for S-phase entry. Importantly, however, pRb/p107 double-null mouse embryonic fibroblasts lacked serum requirement in mid-G1-phase but still required cell anchorage until late G1-phase to enter S-phase. Our results indicate that pRb and p107 do not constitute the last control point for extracellular factors during G1-phase progression, and they functionally separate the requirements for serum and cell anchorage in terms of involved cell cycle components.

Normal tissue cells need extracellular stimuli by growth factors and by integrin-mediated cell anchorage to the extracellular matrix to proliferate, whereas loss of these requirements is a hallmark of tumor progression. Cells that lack growth factors and/or cell anchorage arrest in the G1-phase of the cell cycle. The G1-phase is controlled by several cyclin-dependent kinases (Cdks),1 of which the cyclin D-Cdk4/6 complex is the first to be present (1–3). The cyclin D-Cdk4/6 complex phosphorylates, and thereby inactivates, members of the retinoblastoma susceptibility gene product (pRb) family of pocket proteins, consisting of pRb, p107, and p130 (4). Active pRb blocks G1-phase progression by binding and inhibiting E2F transcription factors (5). The inhibitory phosphorylation of pRb by cyclin D-Cdk4/6 results in the activation of E2F and subsequent expression of proteins promoting cell cycle progression, including cyclin E and cyclin A. The subsequent cyclin E-Cdk2 activity might phosphorylate pRb on additional phosphorylation sites, resulting in a complete inhibition of pRb (3, 6, 7). Inactivation of pRb has also been suggested as the event in the G1-phase that corresponds to passage through the restriction point, a point after which cells are independent of extracellular mitogenic stimuli to enter S-phase (2, 8, 9). Growth factors and cell anchorage jointly stimulate cell proliferation by regulation of cell cycle G1-phase progression (10–12). Signals from various growth factors and from cell anchorage converge on cyclin D-Cdk4/6 activity (13, 14), mainly mediated by the regulation of levels and timing of increased cyclin D1 protein that constitute the rate-limiting step in cyclin D-Cdk4/6 complex formation (14, 15). Cells that are denied either growth factors or cell anchorage fail to induce and to keep sustained levels of cyclin D1 and thereby get arrested in early to mid G1-phase. Taken together with reports demonstrating that signals from both growth factors and cell anchorage also regulate Cdk2-inhibitor protein levels and cyclin E-Cdk2 activity (16–21), these facts support the hypothesis that signals from growth factors and anchorage jointly control the cell cycle G1-phase progression. According to this model, growth factors and anchorage stimulate G1-phase progression jointly through the same downstream cell cycle targets, including through inactivation of pRb and the restriction point (10, 14). However, although many cell cycle G1-phase components can be regulated by both growth factors and cell anchorage, it is unclear whether growth factors and cell anchorage functionally regulate the G1-phase through an identical set of cell cycle components or whether there are distinct sets of functional cell cycle targets. Therefore, we tested whether the requirements of growth factors (serum) and cell anchorage may be separate during the G1-phase progression in primary fibroblasts.

We found that the requirements of serum and cell anchorage can be functionally separated both in time and with regard to involved cell cycle components during the G1-phase of primary human and mouse fibroblasts, where serum was required until mid-G1-phase, whereas cell anchorage was needed until late G1-phase. Importantly, the final anchorage-dependent G1-phase control was intact in pRb/p107 double-null mouse embryonic fibroblasts (MEFs), whereas these cells lacked the normal serum requirement. We conclude that the final functional G1-phase control by cell anchorage is distinct from the serum control, in that it occurs after and independent of normal serum control, normal cyclin D-associated kinase activity, pRb, and p107.
Fig. 1. Temporal separation of the requirements for serum and anchorage in the fibroblast cell cycle G1-phase. a, quiescent, primary NHDF were released from G0-phase into G1-phase. The time until serum (○) or anchorage (□) was needed to achieve competence for S-phase entry (left axis) as well as the time of S-phase entry (□) (right axis) were examined and calculated as described under “Experimental Procedures.” The graph is representative of five independent experiments. b–e, G0-synchronized NHDF were restarted and allowed to progress through the G1-phase in the presence (b) or absence (c) of cell anchorage. Cells were harvested at the indicated time points and subjected to Western blot analysis of cyclin D1, cyclin E, pRb, and cyclin A, as described under “Experimental Procedures.” β-actin was used as a control for equal loading. d, quiescent NHDF were restarted, and cyclin D1/D2 (upper panel) and cyclin E (lower panel) were immunoprecipitated followed by in vitro kinase assays analyzing associated Cdk-activities. The displayed gels are representative among three independent experiments. e, quantification of the cyclin D-associated (○) and cyclin E-associated (□) kinase activities shown in d.

EXPERIMENTAL PROCEDURES

Cells and Cell Cycle Experiment—Primary normal human dermal fibroblasts (NHDF) (BioWhittaker) from two different donors, primary human neonatal foreskin BJ-fibroblasts (kindly provided by Dr. J. W. Shaye, NIH 3T3 mouse fibroblasts (American Type Culture Collection), wild-type, pRb-null, p107-null, and pRb/p107 double-null primary MEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal calf serum (FCS) (BioWhittaker). 50% confluent NHDF were restarted and allowed to progress through the G1-phase in the presence (b) or absence (c) of serum and anchorage to enter S-phase. NHDF were restarted, and cyclin D1/D2 and cyclin E-associated Cdk-activities. The displayed gels are representative among three independent experiments. e, quantification of the cyclin D-associated (○) and cyclin E-associated (□) kinase activities shown in d.

Immunoblotting—Cell lysates were obtained as described (24) by freeze-thawing in lysis buffer followed by centrifugation; 10 or 30 µg of total protein was analyzed by immunoblotting as described previously (20). The following mouse monoclonal antibodies were used: anti-human cyclin D1 mAb (C004, Calbiochem), anti-cyclin E mAb HE111 (Research Diagnostics Inc.), anti-cyclin A mAb H-432 (Santa Cruz Biotechnology). Secondary antibodies were obtained from Jackson ImmunoResearch.

RESULTS

Temporal Separation of the Requirements for Serum and Anchorage in the Fibroblast Cell Cycle G1-phase—Although several critical cell cycle components, including cyclin D and cyclin E are regulated both by growth factors and by cell anchorage, it is not yet clear whether the entire regulation of the G1-phase by extracellular factors occurs jointly or if the requirements for growth factors and cell anchorage can be separated. To circumvent the fact that late G1-phase control depends on early G1-phase signaling, we used the model of the restriction point as a tool to examine whether the final requirements for growth factors and cell anchorage occurred jointly or could be separated in time. We compared the period of time after G0-release that NHDFs needed cell anchorage and serum

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to be able to enter S-phase. NHDF cells were synchronized in G0 by starvation and then released into the G1-phase by re-seeding in serum-containing media. At different time points, serum or attachment was then removed from the cells, and the number of cells that had entered S-phase after a total of 30 h was quantified (Fig. 1a–c). Previous studies have shown that fibroblasts need growth factors for S-phase entry for most cells. However, surprisingly, NHDF displayed a normal G1-phase progression, we analyzed cyclins, cyclin-dependent kinase activities, and the phosphorylation status of pRb in these cells after release from quiescence, with or without cell anchorage. As shown in Fig. 1, a and c, cyclin D1, cyclin E, cyclin A, and pRb-phosphorylation were regulated by cell anchorage in our NHDF and by kinetics similar to those previously reported for various fibroblasts (2, 18, 27). Furthermore, the increased levels of cyclin D-associated kinase activity at 10–14 h occurred at the time point of final control by serum, whereas the peak in cyclin E-associated kinase activity at 14–18 h occurred during the final control by cell anchorage (Fig. 1, a, d, and e).

**Genetic Targeting of pRb and p107 Functionally Separates the Requirements for Serum and Anchorage**—Members of the retinoblastoma susceptibility gene product (pRb) family of pocket proteins are central for G1-phase control, and inactivation of these proteins is regulated both by growth factors and anchorage (14). To examine whether inactivation of the pRb-family of pocket proteins influenced the requirements for serum and/or anchorage, we analyzed for what length of time serum and anchorage were needed to enter S-phase for primary MEFs isolated from mice where pRb and/or p107 were genetically targeted. As shown in Fig. 2, wild-type, pRb−/−, and p107−/− MEFs all required anchorage for a longer time than serum to enter S-phase; therefore, we concluded that the functional control for serum and anchorage in the G1-phase were
distinct in time in three additional primary fibroblast cells. In contrast, pRb/p107-null MEFs lacked a normal serum requirement in mid-G1-phase, because 30 min of serum stimulation after restart was sufficient for these cells to be able to proceed into S-phase (Fig. 2d). Importantly, although the pRb/p107-null MEFs lacked the regular control by serum, these cells retained an intact anchorage control in the late G1-phase (Fig. 2d). This separates the cell anchorage-dependent control from the serum-dependent control not only in time but also with regards to the mediating cell cycle components. **Cell Anchorage Regulates Cyclin E-associated Kinase Activity Independent of Normal Cyclin D, pRb, and p107**—The peak of cyclin E-Cdk2 activity correlated in time to the final requirement for cell anchorage in the late G1-phase (Fig. 1). In addition, cyclin E-associated kinase activity was regulated by cell anchorage in fibroblasts after release from quiescence (Fig. 2e), which confirms previous studies (16, 18, 27). However, given that cyclin D1 levels are tightly regulated by cell anchorage (18, 28, 29), and that cyclin D-Cdk4/6 activity is a pre-requisite for subsequent cyclin E-Cdk2 activity (1), results obtained so far showing cell anchorage-dependent regulation of cyclin E-Cdk2 activity might just reflect cell anchorage-dependent cyclin D regulation. To determine whether cyclin E-associated kinase activity could be regulated by cell anchorage also independently of the regular induction of cyclin D-associated kinase activity in mid G1-phase, we examined cyclin E-coupled activities in the pRb/p107 double-null MEFs that lacked detectable cyclin D-associated kinase activity (Fig. 2e, upper panel). Interestingly, the pRb/p107-null MEFs still required cell anchorage to maintain cyclin E-dependent kinase activity, because we observed a dramatic decrease of cyclin E-dependent kinase activity when these cells were set in suspension (Fig. 2e, lower panel). The residual cyclin E-associated kinase activity in suspended pRb/p107-null MEFs most likely originated from the fraction of cells progressing through the G1-phase independently of cell attachment (data not shown). However, cell anchorage did not regulate cyclin E protein levels in pRb/p107-null MEFs (Fig. 2f), indicating that the regulation of cyclin E-Cdk2 activity occurs at another level. Interestingly, we found markedly higher levels of the Cdk2-inhibitor p27KIP1 in suspended pRb/p107 double-null MEFs compared with attached cells, but no difference for another Cdk2-inhibitor, p21CIP1 (Fig. 2f).

**DISCUSSION**

Our study demonstrates that normal primary human and mouse fibroblasts require cell anchorage for a longer time than serum during the cell cycle G1-phase to be able to enter S-phase. Wild-type, pRb+/−, and p107+/− MEFs had normal requirements for serum and also required cell anchorage for a longer time than serum. This result confirms previous findings that germline inactivation of pRb alone does not alter the serum-dependent control in MEFs (30). However, a recent study indicates that MEFs with acute loss of pRb and p107 do not constitutively require serum in mid-G1-phase (26). Applied in this model, our results indicate that events involving pRb and p107 may regulate the mid-G1-phase window, whereas other factors, such as p130, may control the G1 to G1/phase transition (4).

Importantly, although the pRb/p107-null MEFs lacked a regular serum requirement, they retained an intact anchorage-dependent control until late G1-phase. This finding suggests that, whereas the serum control in these cells seems to be dependent on events involving the pocket proteins pRb and p107, the final anchorage-dependent control is located after and independent of these events. These results functionally separate the requirements for serum and cell anchorage in terms of involved cell cycle components. These results also indicate that inactivation of pRb and p107 does not constitute the final point of control in the G1-phase. Furthermore, the existence of a distinct cell anchorage-dependent control in the late G1-phase, in addition to the mid-G1-control, highlights the importance for a cell to ensure that cellular proliferation occurs only in a proper extracellular matrix context.

Cyclin E-Cdk2 activity is considered crucial for S-phase entry (32), although a recent study indicates that it is possible that cyclin A-Cdk2 may compensate for cyclin E in cyclin E-deficient mouse cells (33). Given that pRb/p107-null MEFs fail to induce cyclin E-associated kinase activity in suspension, the G1-phase progression in pRb/p107-null MEFs must be controlled by anchorage at or before cyclin E-Cdk2 activity. However, we found that this control by cell anchorage seems to be independent of normal cyclin D-associated kinase activity, pRb, and p107, which are implicated in the serum-dependent control (23, 31). Considering (i) that cyclin E has been described as emerg-
We show for the first time that the requirements for serum and cell anchorage can be functionally separated in time and in terms of involved cell cycle components during the G1-phase of primary fibroblasts. Although the final requirement for serum corresponds to inactivation of pRb and p107 in mid-G1-phase, the requirement for anchorage is maintained in the late G1-phase independently of normal serum control, normal cyclin D-associated kinase activity, pRb, and p107 (Fig. 5). The anchorage-dependent control of cyclin E-Cdk2 activity in the late G1-phase may involve regulation of cyclin E-Cdk2 activity. These results provide new functional insights into the extracellular control of cell proliferation.

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