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Altered regulation of G_1 cyclins in senescent human diploid fibroblasts: Accumulation of inactive cyclin E–Cdk2 and cyclin D1–Cdk2 complexes

(cell cycle/cellular aging/retinoblastoma protein/phosphorylation)

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ABSTRACT **Senescent human diploid fibroblasts are unable to enter S phase in response to mitogenic stimulation. One of the key deficiencies in mitogen-stimulated senescent cells is their failure to phosphorylate the retinoblastoma protein, which acts as an inhibitor of entry into S phase in its unphosphorylated form. Recent data suggest that cyclin-dependent kinases (Cdks) regulated by G_1 cyclins (D type and E) are responsible for the primary phosphorylation of the retinoblastoma protein prior to the G_1/S boundary. Surprisingly, we found 10- to 15-fold higher constitutive amounts of both cyclin E and cyclin D1 in senescent cells compared to quiescent early-passage cells. Nevertheless, cyclin E-associated kinase activity in senescent cells was very low and did not increase significantly upon mitogenic stimulation even though cyclin E–Cdk2 complexes were abundant. In contrast to early-passage cells in late G_1 phase, senescent cells contained mainly underphosphorylated cyclin E and proportionally more unphosphorylated and inactive Cdk2, perhaps accounting for the low kinase activity. We also show that a majority of the Cdk2 in senescent cells, but not in early-passage cells, was complexed with cyclin D1. Cyclin D1–Cdk2 complexes, severalfold enriched in senescent cells, contained exclusively unphosphorylated Cdk2. Amounts of cyclin A, which ordinarily accumulates in S and G_2 phases, were extremely low in stimulated senescent cells. We suggest that the failure to activate cyclin E–Cdk2 kinase activity in senescent cells may account for the inability of these cells to phosphorylate the retinoblastoma protein in late G_1 phase, which in turn may block the expression of late G_1 genes such as cyclin A that are required for entry into S phase.**

Human diploid fibroblasts have a finite proliferative lifespan at the end of which they are unable to enter S phase in response to mitogenic stimulation even though they remain alive for many months (1). The G_1-phase arrest state of senescent cells has much in common with the G_1-phase arrest state of early passage cells that are quiescent due to either serum deprivation or cell crowding. However, the early-passage quiescent cells can be induced to initiate DNA synthesis by mitogenic stimulation or subcultivation, whereas the senescent cells cannot. Previous studies have shown that serum-stimulated senescent cells and serum-stimulated quiescent cells are able to carry out many of the same prereplicative events, e.g., expression of c-myc, c-Ha-ras, and p53 (2, 3). However, the following key events fail to take place properly in serum-stimulated senescent cells: (*i*) senescent cells exhibit greatly reduced expression of the immediate early gene c-*fos* (3), (*ii*) they lack the mRNAs for late G_1 genes such as cyclin A, Cdc2, and proliferating-cell nuclear antigen (4, 5), and (*iii*) they fail to phosphorylate their retinoblastoma protein (pRb) (6). Because most of these events have been shown to be necessary for entry into S phase in cycling cells or mitogen-stimulated quiescent cells (for review, see ref. 7), their deficiency may account for the inability of senescent cells to enter S phase. Consequently, it is important to investigate the basis for the failure of senescent cells to carry out these prereplicative events.

Control of cell proliferation in eukaryotes from yeast to humans involves the regulated synthesis, activation, and degradation of a family of cyclins that act as the regulatory subunits of protein kinases termed cyclin-dependent kinases (Cdks; for review, see ref. 8). Recent data suggest that a class of cyclins implicated in G_1-phase progression (9, 10) may be responsible for the phosphorylation of pRb in middle to late G_1 phase (11–13), making it important to determine whether senescent cells synthesize and activate these complexes. In addition, as human fibroblasts approach the end of their lifespan, they become enlarged and their G_1 periods lengthen (14), which is a phenotype shared by *Saccharomyces cerevisiae* cells that are deficient in the accumulation of G_1 cyclins (15). These observations, therefore, suggested that senescent human fibroblasts might be deficient in their accumulation and/or activation of G_1 cyclins.

MATERIALS AND METHODS

Cell Culture. IMR-90 human fetal lung fibroblasts were cultured as described (16). Young cells [population doubling (PD) 20–32] were made quiescent by growth to high cell density. Senescent cells (PD 63–70) were cultured in growth medium containing 10% (vol/vol) fetal bovine serum until they reached the point where they could no longer achieve 1 PD in 3 weeks with weekly refeedings of sparse cultures. Serum-deprived senescent or quiescent cells were incubated for an additional 4 days in medium containing 0.1% fetal bovine serum. Senescent or quiescent cultures had <5% labeled nuclei after a 24-h period of [^{3}H]thymidine incorporation (16).

Analysis of Steady-State Amount of mRNA. Poly(A)$^+$ RNA was prepared and analyzed on Northern blots as described for cyclin A and β-actin (4). Blots were also probed for cyclins D1 and E, using their respective cDNAs (17) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using pHcGAP (American Type Culture Collection). Quantitation was carried out with an Ambis radioanalytic analyzer or by densitometry of autoradiographs of the blots. All data were normalized to both β-actin and GAPDH to give the ranges of values cited in the text.

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Abbreviations: pRb, retinoblastoma protein; PD, population doubling; GST, glutathionine *S*-transferase; R point, restriction point; Cdk, cyclin-dependent kinase.

Immunoblot Analysis and Immunoprecipitation. Frozen pellets of cells (harvested by trypsinization) were lysed in ice-cold RIPA buffer (18). Proteins were fractionated on 11% gels by SDS/PAGE, immunoblotted, detected, and quantitated as described (18). Cyclin and Cdk complexes were immunoprecipitated from whole-cell lysates as described (18) (except for the additional use of a cyclin E-specific hybridoma supernatant, HE111) and were used for either anti-PSTAIRE immunoblots or histone H1 kinase assays (18). For double immunoprecipitation experiments, cyclin–Cdk complexes (first immunoprecipitation) were dissociated by incubation in 1% SDS for 30 min at 37°C, diluted 1:10 in TNET (50 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1% Triton X-100), and then immunoprecipitated with either Cdk2-specific rabbit polyclonal antibody (Upstate Biotechnology) or Cdc2-specific rabbit antiserum (C. McGowan, Scripps).

Cdc25 Phosphatase Treatment. Washed immunoprecipitates were incubated for 30 min at 30°C in 100 μl of phosphatase buffer [25 mM imidiazole, pH 7.2/2 mM EDTA/10 mM dithiothreitol/bovine serum albumin (0.1 mg/ml)] with and without glutathionine *S*-transferase (GST)–Cdc25 at 100 μg/ml (a gift from J. Millar and C. McGowan, Scripps). This treatment completely dephosphorylated cyclin B1-associated Cdc2 (ref. 19 and unpublished observation).

RESULTS AND DISCUSSION

Overabundance and Altered Regulation of Cyclin D1 and Cyclin E mRNA in Senescent Human Fibroblasts. Serum stimulation induced a 3- to 4-fold increase of cyclin D1 and cyclin E mRNA in quiescent IMR-90 cells at least 3–6 h before the stimulated quiescent cells produced cyclin A mRNA (Fig. 1*A*) and initiated DNA synthesis (data not shown). A similar time course for induction of cyclin D1 gene expression has been reported for Hs68 and WI-38 human fibroblasts (20, 21). Surprisingly, unstimulated senescent IMR-90 had 3–6 times as much cyclin D1 mRNA and 2–4 times as much cyclin E mRNA as did unstimulated quiescent cells (Fig. 1*B*). Serum stimulation caused an average 1.4-fold increase in the mRNAs for both cyclin D1 and cyclin E in senescent cells. The overabundance of both G_1 cyclin mRNAs in unstimulated senescent cells suggests that their down-regulation may be deficient in senescent cells. In contrast, senescent cells lacked cyclin A and cyclin B1 transcripts both before and after stimulation (Fig. 1*A* and ref. 4).

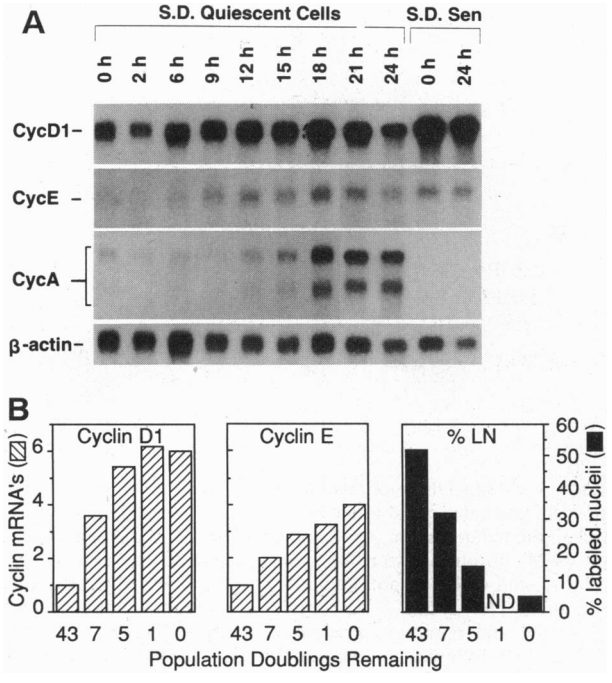

FIG. 1. Steady-state amounts of cyclin D1, E, and A mRNAs in serum-stimulated quiescent and senescent cells. (*A*) Northern blots of poly(A)$^+$ mRNA prepared from IMR-90 cells at various times after serum stimulation were probed sequentially for cyclin D1, E, and A and β-actin transcripts. (*B*) Cyclin D1 and E mRNAs in unstimulated late-passage quiescent and senescent cells, relative to early-passage quiescent cells (43 PD remaining). %LN, [^{3}H]thymidine labeling index for a 24-h period after serum stimulation; S.D., serum deprived; Sen, senescent; Cyc, cyclin; ND, not done.

Recently, Lucibello *et al.* (21) have reported similar increases in cyclin D1 and cyclin E transcripts in senescent WI-38 cells. In contrast, Won *et al.* (20) found that cyclin D1 mRNA levels were low in unstimulated cultures of both early- and late-passage Hs68 cells; after serum stimulation, there was more cyclin D1 mRNA in early-passage cells than in late-passage cells. Since the cells used by Won *et al.* (20) were not described as fully senescent, we investigated whether an overabundance of transcripts for cyclins D1 and E was unique to the fully senescent state. Our data indicate that the overabundance of cyclin D1 and cyclin E transcripts is attained progressively with the declining proliferative capacity of late-passage cultures (Fig. 1*B*).

Accumulation of G_1 Cyclins in Stimulated Quiescent and Senescent Cells. The observed abundance of G_1 cyclin mRNAs in senescent cells prompted us to analyze the pattern of cyclin protein accumulation in serum-stimulated quiescent and senescent IMR-90 cells. Serum stimulation of quiescent early-passage cells resulted in a dramatic (15-fold) accumulation of cyclin D1 by mid-G_1 phase and of cyclin A by G_2 phase, while cyclin E underwent a relatively modest (3- to 4-fold) increase by mid- to late G_1 phase (Fig. 2*A*). In addition, a portion of the cyclin E molecules also showed a characteristic shift toward reduced electrophoretic mobility at these times, suggesting possible post-translational regulation of this cyclin. In studies to be reported elsewhere (V.D., W.K. Kaufman, S.J. Wilson, and S.I.R., unpublished data), we have demonstrated that this shift is a result of phosphorylation of cyclin E.

The regulation of cyclin D1 and cyclin E protein accumulation was altered dramatically in senescent fibroblasts. (*i*) There were high constitutive amounts of both G_1 cyclins in unstimulated senescent cells (10- to 15-fold increases relative to unstimulated quiescent cells; Fig. 2*A*). In contrast, cyclin A levels were extremely low and indistinguishable from amounts observed in quiescent cells. (*ii*) Senescent fibroblasts showed only a modest further accumulation of these cyclins in response to serum stimulation (25–50% for cyclin D1 and 20% for cyclin E). (*iii*) Senescent cells accumulated predominantly unphosphorylated forms of cyclin E before and after mitogen stimulation. Our data suggest that the phosphorylation of cyclin E is associated with the activity of the cyclin E–Cdk2 kinase complex (see below).

Cyclin E-Associated Kinase Activity Is Low in Senescent Cells. Cyclin E immunoprecipitates have a histone H1 kinase activity that is believed to play a role in phosphorylation of pRb and in G_1 progression (10, 11, 18, 23). We therefore compared cyclin E-associated histone H1 kinase activity in serum-stimulated quiescent and senescent cells. (Fig. 2*B*). As expected (see refs. 18 and 23), cyclin E immunoprecipitates prepared from quiescent and early G_1 cells (6 h after stimulation) showed low H1 kinase levels that increased dramatically when the cells reached late G_1 phase (12 h). In senescent cells, however, high levels of cyclin E protein were not paralleled by elevated cyclin E-associated kinase activity, nor did mitogen stimulation cause a significant increase in activity for times up to 36 h after serum stimulation (Fig. 2*B* and data not shown for 30–36 h after stimulation).

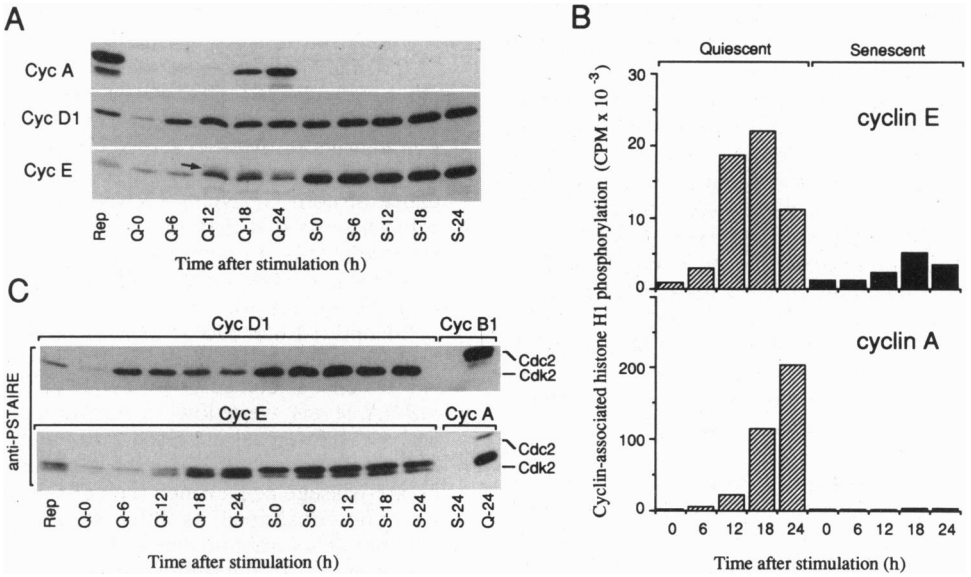

FIG. 2. Analysis of G_1 cyclin protein amounts (*A*), associated histone H1 kinase activity (*B*), and formation of cyclin–Cdk complexes (*C*) in quiescent and senescent human diploid fibroblasts at various times (in hours) after serum stimulation. (*A*) Immunoblots probed sequentially with antibodies to cyclins E, D1, and A. Phosphorylated cyclin E forms are indicated by an arrow. Rep, cycling cells; Q, quiescent cells; S, senescent cells; Cyc, cyclin. (*B*) Histone H1 kinase activity of anti-cyclin E and anti-cyclin A immunoprecipitates from aliquots of the same cell extracts used for *A*. The small amount of kinase activity in the lysate of senescent cells stimulated for 18 h shown is the maximum that we have observed in three experiments. Some of this activity may be attributed to the small fraction of cells (2.5%) in the senescent population that entered S phase in response to serum stimulation. (*C*) Cyclin D1 and E immunoprecipitates prepared as in *B* were analyzed on an immunoblot with anti-PSTAIRE monoclonal antibody (22). Cyclin A and cyclin B1 immunoprecipitates were analyzed for comparison.

Mitogen-stimulated senescent cells also lacked cyclin A-associated kinase activity, consistent with the lack of cyclin A protein in these cells.

The deficiency in cyclin E-associated kinase activity in stimulated senescent cells may account for the lack of phosphorylation of pRb in late G_1 phase in these cells. Although the kinases normally responsible for pRb phosphorylation are not known with certainty, cyclin A-, cyclin D2-, and cyclin E-associated protein kinases are good candidates for this function because ectopic expression of cyclins A, D2, or E stimulated phosphorylation and inactivation of pRb *in vivo* (11, 13). However, IMR-90 cells lack cyclin D2 (9) and acquire phosphorylated pRb prior to activation of the bulk of their cyclin A-associated kinase activity (7), making a cyclin E-associated kinase the most likely candidate for the late-G_1-phase pRb kinase in these cells. In addition, we have observed that the kinetics of activation of the cyclin E-associated kinase correlate well with the kinetics of phosphorylation of pRb in IMR-90 cells (data not shown and ref. 7).

Cyclin E Forms an Inactive Complex with Cdk2 in Senescent Cells. The low amounts of cyclin E-associated kinase activity in senescent cells could reflect either a lack of complex formation between cyclin E and its kinase partner, Cdk2 (18, 23), or the presence of an inhibitory modification of either component. As shown earlier, cyclin E binds to two major SDS/PAGE mobility forms of Cdk2 (Fig. 3*A* and ref. 18). The slower mobility form (enriched in quiescent and early-G_1-phase cells, Fig. 2*C*) corresponds to unphosphorylated and inactive Cdk2, whereas the faster mobility form (enriched in late-G_1- and S-phase cells) is phosphorylated on Thr-160 and is the potentially active form (18, 24). Active cyclin E kinase complexes (from quiescent cells stimulated for 12 and 18 h) contain predominantly Thr-160-phosphorylated Cdk2, as do cyclin A–Cdk2 complexes (Figs. 2*C* and 3*A*). Surprisingly, we found abundant cyclin E–Cdk2 complexes in senescent cells. Although these complexes contained predominantly unphosphorylated Cdk2, the absolute amount of Thr-160-phosphorylated Cdk2 was comparable to that found in stimulated quiescent cells (Fig. 2*C*, quiescent vs. senescent cells stimulated for 12 h), whereas the kinase activity of these same immunocomplexes was only 11% as high in senescent cells

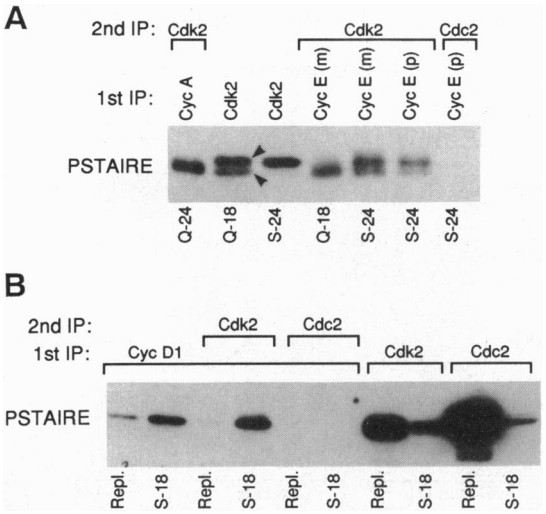

FIG. 3. Association of cyclin E and cyclin D1 with Cdk2 in quiescent and senescent human fibroblasts. (*A*) Lysates from 18- and 24-h stimulated quiescent (Q-18 and Q-24) and 24-h stimulated senescent (S-24) fibroblasts were immunoprecipitated (1st IP) with anti-cyclin A, anti-Cdk2, or anti-cyclin E (m, monoclonal; p, polyclonal) antibodies. The cyclin A and cyclin E immune complexes were dissociated and reimmunoprecipitated with antibodies to Cdk2 or Cdc2 (2nd IP) followed by immunoblot analyses with anti-PSTAIRE antibody. Arrowheads mark the active (faster migrating) and inactive (slower migrating) forms of the Cdk2 doublet. (*B*) Anti-cyclin D1 immunoprecipitates (1st IP) prepared from lysates of young replicating cells (Repl.) and 18-h stimulated senescent cells (S-18) were divided into three equal parts and left untreated or reimmunoprecipitated with antibodies to Cdk2 or Cdc2 (2nd IP), prior to immunoblot analysis with anti-PSTAIRE antibody. Anti-Cdk2 and anti-Cdc2 immune complexes prepared from equal amounts of the original cell lysates are also shown. Each of the lanes on the immunoblot derives from the same initial amount of cell lysate protein.

stimulated for 12 h as in quiescent cells stimulated for 12 h (Fig. 2*B*). Hence, the lack of cyclin E-associated kinase activity in senescent cells is not primarily a failure to bind the potentially active form of Cdk2. These data also demonstrate the ability of cyclin E to bind significant amounts of unphosphorylated Cdk2, contrary to the suggestion of Koff *et al.* (25).

Our results also shed more light on the data reported recently by Lucibello *et al.* (21) who detected low cyclin E-associated H1 kinase activity in unstimulated senescent cells. Based on an apparent lack of ^{35}S-labeled Cdk2 in serum-stimulated senescent cells and a failure to examine directly the cyclin E immunoprecipitates, they concluded that in senescent cells, cyclin E cannot associate with its kinase partner. We clearly showed that this is not the case and that both cyclin E and cyclin D1 (see below) form abundant complexes with Cdk2, even though the steady-state levels of Cdk2 are reduced by about half in senescent cells (Fig. 3*A*, compare Cdk2 first immunoprecipitation of 18-h stimulated quiescent cells and 24-h stimulated senescent cells; and ref. 7). Interestingly, the amount of Cdk2 in both stimulated senescent and quiescent cells is much less than in replicating young cells, i.e., ≈10 and 20%, respectively (Fig. 3*B* and data not shown).

Cyclin E–Cdk2 Complexes from Senescent Cells Are Not Activated by Cdc25 Phosphatase *in Vitro*. Because the Thr-160-phosphorylated Cdk2 associated with cyclin E in senescent cells could be inhibited by phosphorylation on Tyr-15 and possibly Thr-14 (24), we attempted to activate these cyclin E immunocomplexes with bacterially produced Cdc25 (19). Cdc25 is a phosphatase that promotes kinase activity of Cdc2 (26) and Cdk2 (24, 27) by dephosphorylating Tyr-15 and Thr-14. As shown in Fig. 4, Cdc25 failed to increase the H1 kinase activity of cyclin E–Cdk2 complexes from stimulated senescent cells (S-12 h) even though it caused a 50% increase in the activity of cyclin E–Cdk2 complexes from 12-h stimulated quiescent cells and a 200% increase in the activity of cyclin B1 immunoprecipitates from asynchronously replicating cells, which contain both active and inactive forms of Cdc2. Since most of the cyclin E–Cdk2 complexes from the 12-h stimulated quiescent cells may already have been in the active state, a relatively modest increase in Cdk2 activation was not unexpected in this case. Similar treatment of cyclin E immune complexes prepared from thymidine-blocked HeLa cells gave rise to a dramatic increase (400%) of H1 kinase activity, showing that Cdc25 can efficiently activate Cdk2 bound to cyclin E (Fig. 4). We conclude that the low kinase activity of cyclin E–Cdk2 complexes in senescent cells is not due to inhibitory phosphorylation on Tyr-15 and Thr-14 but rather occurs by an as yet unknown inhibitory mechanism.

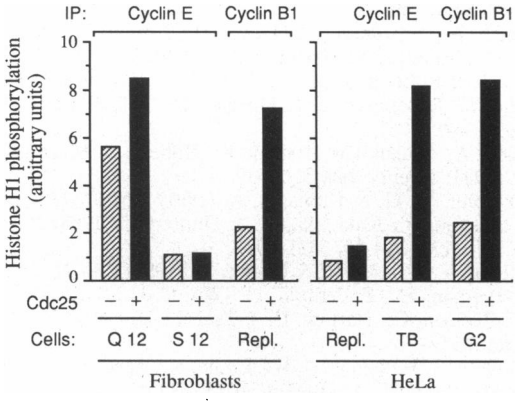

FIG. 4. Cdc25 phosphatase treatment of inactive cyclin E–Cdk2 kinase complexes from senescent cells. Cyclin E–Cdk2 complexes, prepared as in Fig. 2*B* from 12-h-stimulated quiescent (Q12) and senescent (S12) IMR-90 fibroblasts and from replicating (Repl.) and thymidine-blocked (TB) HeLa cells, were incubated with or without GST–Cdc25 phosphatase prior to assay for histone H1 kinase activity. Cyclin B1 immunoprecipitates from replicating IMR-90 and G_2-enriched HeLa cell populations were subjected to the same treatment. The H1 kinase activity in these experiments is normalized to show the relative increase upon phosphatase treatment.

A deficiency in the phosphorylated forms of cyclin E is the primary difference that we have observed in the composition of cyclin E–Cdk2 complexes in stimulated senescent cells vs. stimulated quiescent cells. Since phosphorylated cyclin E appears concommitantly with increased H1 kinase activity of cyclin E–Cdk2 complexes in stimulated quiescent cells, these data suggest the possibility that (*i*) cyclin E phosphorylation is necessary for the activity of cyclin E–Cdk2 complexes and (*ii*) failure to phosphorylate cyclin E is a contributing factor in the lack of cyclin E–Cdk2 kinase activity in senescent cells. However, it is also possible that phosphorylation of cyclin E is an effect, rather than a cause, of activation of the cyclin E–Cdk2 kinase.

Cyclin D1–Unphosphorylated Cdk2 Complexes: Transient Accumulation in Stimulated Quiescent Cells vs. Overabundance in Senescent Cells. Cyclin D1-associated protein kinase activity has not been detected in mammalian cell lysates (12, 13, 21) even though cyclin D1 associates with Cdk2, Cdk4, and Cdk5 (12, 28). Consequently, we could not determine whether cyclin D1 present in senescent cells was associated with an active kinase. However, by immunoblot analysis of cyclin D1 immunoprecipitates probed with a PSTAIRE antibody, we detected a serum-inducible and periodic (maximal in stimulated quiescent cells at 6 h and decreased at 24 h) association of cyclin D1 with a Cdk that has the same mobility as Cdk2 (Fig. 2*C*), whereas Cdk5 migrates more rapidly than Cdk2 (28) and Cdk4 does not react with anti-PSTAIRE antibody (29). Double immunoprecipitation experiments confirmed that unphosphorylated (inactive) Cdk2 accounted for most, if not all, of the PSTAIRE-containing Cdk associated with cyclin D1 in both 18-h stimulated quiescent cells (data not shown) and 18-h stimulated senescent cells (Fig. 3*B*). Moreover, since senescent cells are deficient in Cdk4 mRNA (21), Cdk2 may be the only cyclin D1-associated kinase in these cells. Interestingly, the amount of Cdk2 complexed to cyclin D1 was 3- to 4-fold greater in stimulated senescent cells than in stimulated quiescent cells, perhaps related to the absence of Cdk4. Furthermore, at least 75% of the total Cdk2 in stimulated senescent cells was complexed with cyclin D1 (Fig. 3*B*), whereas only a minor part associated with cyclin E, the less abundant of the two G_1 cyclins in senescent cells (data not shown).

At this point, we cannot say whether increased accumulation of the cyclin D1–Cdk2 complex adversely affects G_1-phase progression in senescent cells. However, the finding that cyclin D1 forms complexes only with unphosphorylated inactive Cdk2 may explain why cyclin D1 immunoprecipitates from both replicating and senescent fibroblasts failed to phosphorylate histone H1 (unpublished observation), which is normally a good substrate for Cdk2. These results may also explain the observation that insect cells infected with Cdk2 and cyclin D1 failed to phosphorylate a GST–Rb fusion protein, whereas Cdk2 was fully active with all other cyclins tested in this system (13, 30). It is possible that the cyclin D1–Cdk2 complex is not recognized by CAK, a Cdc2/Cdk2-activating kinase that phosphorylates Cdc2 on Thr-161 and Cdk2 on Thr-160 (31).

Do Senescent Cells Fail to Pass the Restriction (R) Point? Investigation of the dynamics of cell cycle control and progression in mammalian fibroblasts has led to the concept of the R point commitment to S phase (32). In yeast, a similarly defined point of the cell cycle termed START has been shown to be regulated at the level of activation of the

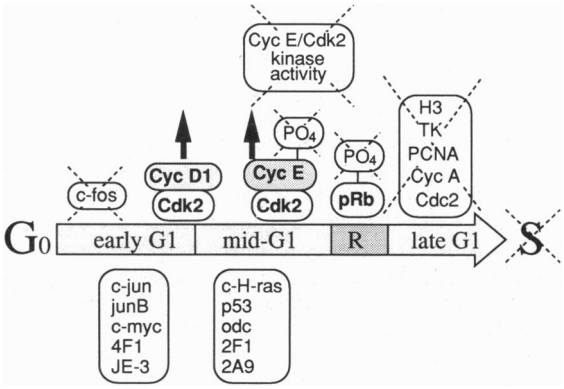

FIG. 5. Summary of serum-inducible events that have been compared in senescent vs. early-passage quiescent human fibroblasts. Serum-stimulated quiescent human fibroblasts carry out all of the diagrammed events, which represent induction of mRNA or protein accumulation unless otherwise indicated. Serum-stimulated senescent cells are deficient in the events crossed out with dashed lines (3–6, 34, 35). Senescent cells also have an overabundance of cyclin D1–Cdk2 and inactive cyclin E–Cdk2 complexes both before and after serum stimulation, as indicated by the solid arrows. Placement of the phosphorylation of pRB at the R point is part of our model but is not proven. H3, histone H3; TK, thymidine kinase; PCNA, proliferating-cell nuclear antigen; odc, ornithine decarboxylase.

Cdc28 kinase by G_1 cyclins (8). The kinetics of accumulation of cyclin E-associated kinase activity in the cell cycle are consistent with an analogous role for this G_1 cyclin in R-point regulation (8). Furthermore, constitutive overexpression of human cyclin E in rat and human fibroblasts shortened the duration of G_1 phase in these cells and decreased their size, suggesting that cyclin E levels are rate-limiting for G_1-phase progression in mammalian cells (10). Thus, the lack of cyclin E-associated kinase activity in stimulated senescent cells could contribute to an inability to pass the R point in these cells. Although the way that cyclin D1 functions is less clear, it also appears to play a critical role in G_1-phase progression (9, 11, 13, 30).

Passage of the R point may be the trigger for the expression of late G_1 genes whose products are necessary for DNA synthesis and mitosis. Phosphorylation of pRb has characteristics consistent with an R-point event because it occurs several hours before S phase and because underphosphorylated pRb has the ability to bind and sequester transcription factors such as E2F, thought to be required for late G_1-phase transcription of a number of S-phase genes (33). If senescent cells are unable to pass the R point, then we would expect them to be preferentially deficient in the expression of late G_1 genes. Although only a handful of late G_1 genes has been compared in senescent and quiescent cells, mitogen-stimulated senescent cells are lacking in transcripts for all of them (Fig. 5). In contrast, stimulated senescent cells are still capable of carrying out most early G_0/G_1 and mid-G_1 cell cycle events. These data suggest the possibility that senescent cells are deficient in a small subset of critical early to mid-G_1 events that are sufficient to impair the activation of the cyclin E–Cdk2 kinase, which in turn would leave pRb in its underphosphorylated pre-R-point inhibitory state, thus blocking late G_1-phase events necessary for entry into S phase. However, another attractive hypothesis is that activation of the cyclin E–Cdk2 kinase is also regulated by a DNA damage checkpoint control mechanism (V.D., *et al.*, unpublished data) that could be responsive to the shortening of chromosomal telomeres that occurs progressively with aging of human fibroblasts (36).

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