Calorie Restriction Influences Cell Cycle Protein Expression and DNA Synthesis during Liver Regeneration

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Calorie restriction without essential nutrient deficiency (calorie restriction, CR) abrogates experimental carcinogenesis and extends healthful life span. To test whether CR influences cell-cycle protein expression during the hepatocellular proliferation induced by 70% partial hepatectomy (PH), BALB/c mice were separated into two groups, fed comparable semi-purified diets for 10 weeks that differed 40% in caloric offering, and were then subjected to PH. When PH was performed, CR mice weighed 36% less than *ad libitum* (AL)-fed mice ($P < 0.01$), but liver-to-body weight ratios were similar. During the regenerative hyperplasia, hepatocytes of CR mice demonstrated evidence of accelerated entrance and passage through G1 and S phases, and an earlier exit from the cell cycle. The first peak of DNA synthesis occurred 6 hr earlier, and the second peak was significantly greater among CR mice with 38% ± 13% bromodeoxyuridine (BrdU)-positive hepatocytes, compared with 14% ± 4% in AL mice ($P < 0.01$). More E2F-1 expression was induced at the hepatic G1/S boundary just prior to each peak of DNA synthesis in regenerating livers of CR mice ($P < 0.01$), and 8 hr earlier among CR mice. More hyperphosphorylated retinoblastoma p110 was detected during hepatic G1 and the G1-S transition among CR mice, coincident with the early hepatocellular proliferative wave. Cyclin A was induced during the first peak of DNA synthesis 4 hr earlier among CR mice, and it continued 4 hr longer in AL mice, indicating an earlier post-replicative exit by hepatocytes in CR mice. p21 was induced during the G1 phase at 4 hr post-PH, and was maximally expressed during and after peak DNA synthesis in both dietary groups. These results indicate that CR influences cell cycle protein expression levels, causing hepatocytes to enter into S phase earlier and exit abruptly from the cell cycle, and they support the premise that CR enhances induced cell responsiveness by influencing cell cycle regulatory controls. [Exp Biol Med Vol. 226(11):1061–1067, 2001]

Key words: cyclin; cyclin-dependent kinase; cyclin-dependent kinase inhibitor; calorie restriction; liver regeneration

The processes required to produce two daughter cells from a preexisting progenitor cell must be precisely accomplished to ensure genomic integrity. Fidelity in the processes that replicate, repair, and segregate the genome is achieved by the coordinated expression and assembly of different kinase holoenzymes composed of a regulatory cyclin, and a catalytic cyclin-dependent kinase (CDK) (1–4). At specific stages of the cell cycle, different cyclin-CDK complexes are assembled and activated that phosphorylate cell cycle-phase specific substrates, triggering events such as DNA replication, nuclear envelope breakdown, spindle assembly, and chromosome segregation.

The most studied G1 cyclin/CDK substrate is the product of the retinoblastoma tumor suppressor gene. Retinoblastoma protein phosphorylation by cyclin/CDKs regulates its interaction with the E2F family of transcription factors. In the quiescent cell resting in G0, the hypophosphorylated form of the retinoblastoma protein (Rb) binds the transcription factor E2F-1, repressing the transcription of genes that contain E2F-1 binding sites in their promoters (5, 6). E2F-1 binding sites are found in the promoters of genes that encode enzymes directly involved in DNA synthesis such as dihydrofolate reductase and DNA polymerase α, as well as other proteins critical for cell cycle progression such as cyclin A, cdc2 (CDK1), and myc and myb family members (1, 7, 8). Hyperphosphorylation of Rb by D-type cyclin/CDKs in mid-G1 results in the disruption of the Rb/E2F-1 complex and the transcription of E2F-dependent genes necessary for cell cycle progression. Other cyclin/CDK complexes are essential for orderly passage through the G2/M phases of the cell cycle. Cyclin A is synthesized in late G1, becomes active as a cyclin A/CDK-2 holoenzyme in S

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phase, and is required for DNA replication (9), but may also modulate some E2F activities (10, 11).

Cells respond biochemically to a plethora of mitogenic and antimitogenic factors, differentiation inducers, and apoptotic signals, which become integrated through signal transduction pathways and feedback loops before committing to enter the S phase. In response to a variety of antimitogenic stimuli, cyclin-dependent kinase inhibitors (CKI) are expressed that modulate the activities of cyclin/CDK complexes. The most extensively characterized CKI is p21, which both inhibits CDK activity (12) and directly inhibits DNA synthesis by complexing with proliferating cell nuclear antigen, an accessory protein to DNA polymerase (13). The p21 gene promoter contains p53-binding sites, and the p53 tumor suppressor protein can induce the production of p21 protein, leading to cell cycle arrest (14). Cells that withdraw from the cell cycle and become either terminally differentiated or senescent express unique CKI profiles that include the p21 protein (15, 16).

The regulation and cell cycle activities of cyclin/CDK complexes, their substrates, and CKIs have been studied primarily in transformed cells and primary fibroblasts in culture. To determine whether the *in vivo* regulation of cell cycle events is associated with similar cyclin/CDK, substrate and CKI gene expressions and activities, the model of hepatocellular regenerative proliferation in response to 70% partial hepatectomy (PH) in rodents has been used (17, 18). Differentiated hepatocytes have a unique capacity to proliferate while performing essential homeostatic functions. The surgical resection of liver lobes results in two coordinated waves of compensatory hepatocellular and stromal cell proliferation within the residual lobe regulated by circulating mitogenic factors, including hepatocyte growth factor, epidermal growth factor, transforming growth factor-α, interleukin-6, tumor necrosis factor-α, insulin, and norepinephrine (19). Morphologically, the pattern of regenerative proliferation follows the metabolic zones of the hepatic acinus, with mitosis occurring first in periportal hepatocytes, and then proceeding to the pericentral areas. Most of the hepatocytes in the residual lobe participate in one or two proliferative events, with the first wave of proliferation in female BALB/c mice represented by a peak in DNA synthesis occurring between the 24th and 36th postoperative hour, and the second, major proliferative wave occurring between 36 and 48 hr after PH (20). With restoration of the hepatic parenchyma within 7–10 days, mitotic rates return to basal levels by mechanisms that include the expression of transforming growth factor-β (19). This model has been used to study the *in vivo* regulation of cell cycle associated genes, including cyclins, CDKs, and CKIs (5, 21–25).

Calorie restriction (CR) to levels 20%–40% less than *ad libitum* consumption without essential nutrient deficiency is the only experimental intervention that slows the intrinsic rate of biological aging, abrogates experimental carcinogenesis, and extends the mean and maximum life span of various species across wide phylogenetic differences (26, 27). Although CR has been shown to enhance immunologic functions (26, 28–30), increase the enzyme-mediated repair of DNA (31), increase free-radical scavenging and reduce oxidative stress (28, 32, 33), increase protein synthesis (34), modulate rates of cell proliferation (35, 36), and alter the rhythms and levels of neuroendocrine secretions (37, 38), a unifying mechanism of the protective effect remains elusive. Translation of this phenomenon to prevention strategies for human cancer and aging disorders will require a clear understanding of the genetic, molecular, cellular, and physiological consequences of CR, and may rely on the identification of surrogate interventions that can mimic the protective effect.

The degree to which a cell faithfully reproduces and selectively withdraws from the cell cycle may influence both the pace of biological aging and the risk for cancer (16). Although an increased rate of cell proliferation *per se* may (39–41) or may not (42–44) be a risk factor for cancer, it is increasingly apparent that defects in the biochemical regulatory controls of ordered cell cycle progression allow for the development of neoplasms (45, 46). The beneficial consequences of CR may be related to its influence on the expression of cell cycle regulatory proteins, a reduction in nonessential cell replication, and an enhanced state of cell responsiveness, but this has not been evaluated.

To test whether the level of calorie intake influences the expression of regulatory cyclin/CDK, substrate, or CKI proteins during cell proliferation, we evaluated the effect of CR on the expression levels of contrasting regulatory proteins that either promote or inhibit cell cycle progression during the compensatory hyperplasia of hepatocytes induced by the partial surgical resection of the liver. Protein expression patterns of E2F-1, Rb p110, cyclin A, and p21 were determined and associated with the bromo-deoxyuridine (BrdU) DNA-labeling index of the proliferating hepatocytes beginning at 20 hr after PH and continuing every 4 hr through the 52nd postoperative hour, and at 72 and 96 hr after PH. Our findings demonstrate that cell cycle protein expressions during liver regeneration are influenced by calorie intake level, and they support the premise that the beneficial effects of CR may be referable to enhanced cell activation and function.

Materials and Methods

Animals. BALB/c, 6-week-old, female mice (The Jackson Laboratory, Bar Harbor, ME), were maintained in accordance with the principles of the *Animal Welfare Act* and the *Guide for the Care and Use of Laboratory Animals*, were used in a protocol approved by the *Institutional Animal Care and Use Committee*, and were separated into two experimental groups. Mice that consumed semipurified diet *ad libitum* were designated group AL. Mice that consumed a similar but CR semipurified diet were designated group CR.

Semipurified Diets. The preparation and composition of the two semipurified diets used have been described

in detail (47) (Table 1). Both diets were low in dietary fat (approximately 6% of total calories) and differed by 40% in the level of total caloric energy available, but were otherwise comparable. The composition of diets was formulated with each incremental increase in CR to ensure an equivalent intake of essential nutrients by all mice, including vitamins, minerals, essential fatty acids, and 30% of calories as protein, while limiting only total dietary calories of the CR mice. Dietary constituents were obtained from ICN Biochemicals (Costa Mesa, CA.). CR was made at increments of 10% fewer dietary calories offered each week relative to the feeding of AL mice. CR mice were offered 60% of the calories offered group AL mice for a period of 10 weeks, when mice were 9–19 weeks of age. Food consumption of pair-fed mice was determined by weighing the food offered and reweighing the remnant food at the end of each feeding interval. Calorie intake was determined as a product of the weight of diet consumed (grams) multiplied by the caloric concentration of the diet offered (calories/gram). Mice were weighed weekly. After 10 weeks of pair-feeding semipurified diets that ensured a 40% difference in caloric offering, mice were subjected to PH.

PH. PH was performed by the resection of hepatic lobes that comprised approximately 70% of the hepatic parenchyma as described by Higgins and Anderson (17). All operations were done during the light phase of the 24-hr cycle between 0900 and 1200 hr. Representative mice (n = 3) each of either group AL or CR were euthanized by carbon dioxide inhalation at intervals 20, 24, 28, 32, 36, 40, 44, 48, 52, 72, and 96 hr post-PH. Total hepatic protein was extracted from the regenerating liver lobe and was analyzed for cell cycle gene expressions. Biopsies of the regenerating liver lobe of some AL or CR mice injected with BrdU were preserved for analysis of DNA synthesis.

Analysis of DNA Synthesis. Representative AL or CR mice (n = 3) that had undergone PH were given 50 mg/kg of BrdU (Amersham Pharmacia Biotech, Piscataway, NJ) by i.p. injection 2 hr prior to euthanasia at 24, 30, 36, 48, 72, or 96 hr post-PH. Comparably spaced sections of each regenerating liver lobe were taken and fixed in 10% buffered formalin, dehydrated, embedded in paraffin, and serially sectioned at 3–5 μm thickness. Each section was deparaffinized, heated in a 0.01 M sodium citrate buffer at pH 6 for 10 min, then cooled at room temperature for 20 min. Immunohistochemical staining for BrdU was performed with the Roche cell proliferation kit (Roche Biochemicals, Indianapolis, IN) and an avidin-biotin complex elite kit (Vector Labs, Burlingame, CA), each according to the manufacturer's recommendations. Slides were counterstained with hematoxylin. The DNA labeling index (DNA-LI) of four sections per animal was determined by counting the number of BrdU-labeled nuclei in a minimum of 300 hepatocytes per section, and was expressed as a percentage of labeled nuclei per 100 cells.

Table I. Composition of Diets

Constituent	*Ad libitum*		40% CR	
	g	kcal	g	kcal
Sucrose	47.25	189.0	26.51	106.05
Glycerol	16.0	64.0	8.98	35.91
Casein	29.4	117.6	17.64	70.56
Methionine	0.6	2.4	0.36	1.44
Safflower oil	2.0	18.0	2.0	18.0
AIN vitamin mix	1.0	3.95	1.0	3.95
AIN mineral mix	3.5	1.65	3.5	1.65
Inositol	0.05	0.2	0.05	0.2
Choline bitartrate	0.2	0.8	0.2	0.8
Total	100.0	397.6	60.24	238.56
Energy (kcal/g)	3.98		3.96	
Protein/total (kcal)	0.302		0.302	
Carbohydrate/total (kcal)	0.636		0.595	
Fat/total (kcal)	0.045		0.075	

Extraction and Analysis of Liver Protein. The hypertrophic, regenerating liver lobe was homogenized on ice in a modified NP-40 buffer (250 mM NaCl, 50 mM Hepes, 5 mM NaF, 0.1% NP-40, and 1 mM DTT) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO). Liver homogenates were vortexed and clarified by centrifugation once at 9,000 rpm for 10 min, and the supernatant was collected and centrifuged at 14,000 rpm for 15 min. Protein concentrations were determined using the Bradford reagent, and aliquots of the supernatant were stored at −80° C until analyzed.

For Western analysis, 50 μg of denatured hepatic protein and an equal volume of 2× Lammeli sample buffer was added to each lane and was size fractionated in 6%, 10%, or 15% polyacrylamide gels, transferred to Hybond-ECL nitrocellulose (Amersham Pharmacia Biotech), and detected using ECL Chemiluminescence (Amersham Pharmacia Biotech). Antibodies used in Western analysis were a rabbit polyclonal anti-cyclin A (#AB-5, Neomarkers, Fremont, CA.), rabbit polyclonal anti-E2F-1 (#SC193, Santa Cruz Biotechnologies, Santa Cruz, CA.), mouse monoclonal anti-Rb p110 (#14001A, PharMingen, San Diego, CA.), mouse monoclonal anti-p21 (#65951A, PharMingen), horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech), and horse radish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech).

Statistical Analysis. Differences in mean caloric intake, body weight, DNA-LI, or densitometric scannings of band densities in Western analyses of cell cycle hepatocellular protein expression during liver regeneration were determined between AL and CR mice using either an analysis of variance or student's t test and were considered significant when $P < 0.01$.

Results

Physical Parameters. When 9–19 weeks of age, CR mice were offered 40% fewer calories and consumed a mean 10.4 kcal/day, or 34% fewer calories consumed compared with AL mice, who consumed a mean 15.8 kcal/day ($P < 0.01$). When PH was performed at 19 weeks of age, the

mean body weight of CR mice was 15.2 ± 0.47 g, and was significantly 36% less than the 23.7 ± 1.06 g mean body weight of age-matched AL mice ($P < 0.01$; Fig. 1). Regardless, mean liver-to-body weight ratios of 19-week-old AL or CR mice were comparable at PH.

Regenerative DNA Synthesis. The patterns and levels of DNA synthesis during hepatic regeneration were determined morphologically by determining the percentage of BrdU-labeled hepatic nuclei in the regenerating livers of three AL or three CR mice each at 24, 30, 36, 48, 72, and 96 hr after PH. The earliest BrdU nuclear labeling was observed in periportal hepatocytes (zone 1 of the hepatic acinus) of CR mice. The first wave of hepatocellular proliferation occurred 6 hr earlier in CR mice, and at 30 hr after PH, with 15% of hepatocellular nuclei labeled with BrdU, compared with fewer than 1% in AL mice (Fig. 2). The first proliferative wave did not occur in AL mice until 36 hr after PH. The second proliferative wave occurred 48 hr after PH in both dietary cohorts, and was significantly greater among CR mice with 38% ± 13% of hepatocytes in S phase compared with 14% ± 4% in AL mice ($P < 0.01$). The mean hepatic DNA-LI of CR mice was greater than that of AL mice at each interval of assessment except at 36 hr post-PH, with 8% of hepatocytes in CR mice labeled compared with 25% in AL mice, although this difference in mean DNA-LI lacked statistical strength. At 72 hr after PH, the mean DNA-LI was similar for each dietary group, with approximately 11% BrdU-labeled hepatocytes. Fewer than 1% of hepatocytes were in S phase in liver biopsies from either dietary cohort at 96 hr post-PH, indicating that most hepatocytes had exited the cell cycle by the fourth postoperative day.

Expression of Cell Cycle Regulatory Proteins. To assess the influence of dietary calories on E2F-1, Rb p110, cyclin A, and p21 protein expression during liver regeneration, Western blot analysis was performed on extracts from homogenates of liver tissue collected prior to 70% surgical resection and at 20, 24, 28, 32, 36, 40, 44, 48, 52, 72, and

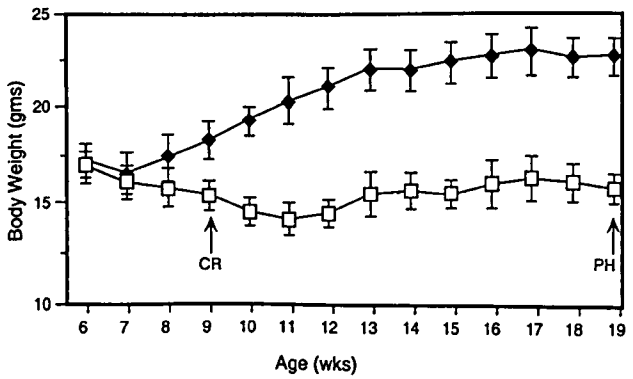

Figure 1. Mean ± SD body weights of BALB/c female mice fed semipurified diets, either AL (♦) or CR (□). CR mice were fed a comparable diet, but with a 10% reduction in caloric offering per week for 4 weeks, and 40% fewer calories (CR, arrow) were offered for 10 weeks from the nine to the 19th week of age until PH (arrow). Mean body weights of CR mice were significantly less than that of AL mice when ≥9 weeks old ($P < 0.01$).

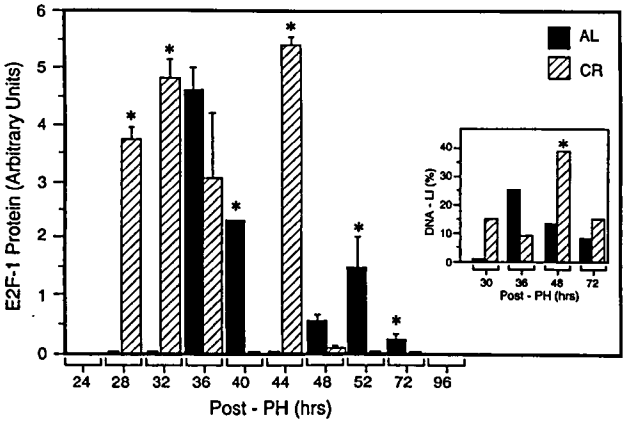

Figure 2. Mean hepatocellular expression of E2F-1 protein during liver regeneration in AL (solid bar) or CR (striped bar) mice. Each bar represents mean ± SD of three individual mice. Ordinate shows arbitrary units of E2F-1 protein expression determined in Western analyses by quantifying bands of E2F-1 protein with a laser densitometer. Inset shows mean hepatocellular DNA-LI of BrdU-labeled regenerating livers. Peak E2F-1 protein expressions and DNA-LIs occurred earlier among CR mice (*$P < 0.01$).

96 hr after PH. Hepatic expression of E2F-1, Rb p110, cyclin A, and p21 proteins was activated by PH, and the pattern and levels of these expressions in representative AL or CR mice are shown in Figures 3 through 6.

No E2F-1 protein was detected in the quiescent liver 0, 20, or 24 hr following PH. Two principle peaks of E2F-1 protein expression were detected in the regenerating livers of each dietary group, with both peaks of E2F-1 expression occurring earlier in CR mice, and each peak of E2F-1 expression preceding the two peaks in DNA-LI described above (Figs. 2 and 3). Hepatic expression of E2F-1 protein was first detected in CR mice, coincident with the first proliferative wave, 8 hr earlier at 28 hr compared with 36 hr after PH for AL mice. Hepatic E2F-1 expression was detected at the intervals 28, 32, and 36 hr after PH in 75% of CR mice, in contrast to no detectable expression of E2F-1 protein in livers of AL mice until the 36th postoperative hour. During this first wave of hepatocellular proliferation, mean E2F-1 protein expressions in the regenerating livers of CR mice were significantly greater than that of AL mice 28 and 32 hr after PH ($P < 0.01$), and comparable 36 hr post-PH. The first hepatic expression of E2F-1 protein in AL mice also coincided with the first proliferative wave of regeneration in that cohort, but was delayed until the 36th and

Figure 3. Western blot analysis of E2F-1 protein expressed in livers during the regenerative hyperplasia induced by PH in representative individual AL or CR mice. E2F-1 protein was expressed just prior to and coincident with each of the two hepatocellular proliferative waves of regeneration, and was detected earlier in livers of CR mice.

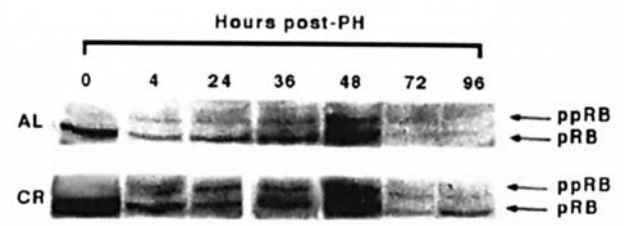

Figure 4. Expression of the hypophosphorylated (pRb) and hyperphosphorylated (ppRb) forms of retinoblastoma p110 protein in livers during regeneration after PH in representative individual AL and CR mice was detected by Western blot analysis. pRb was detected prior to PH, and at each postoperative interval during regenerative hyperplasia. ppRb p110 protein was principally detected during the second proliferative wave at the 48th-hr postoperative interval in both dietary groups.

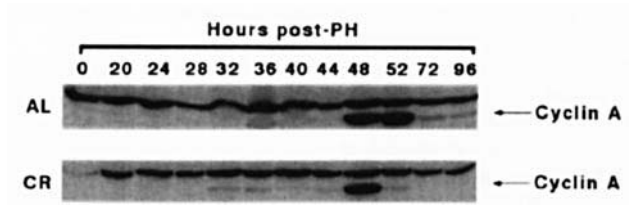

Figure 5. Western blot analysis of cyclin A protein expression in livers of representative individual AL or CR. Cyclin A protein was detected 4 hr earlier during the first proliferative wave in CR mice, it was maximally expressed in mice of both dietary cohorts during the second proliferative wave at the 48th-hr interval, and it extended 4 hr later through the 52nd postoperative interval in AL mice.

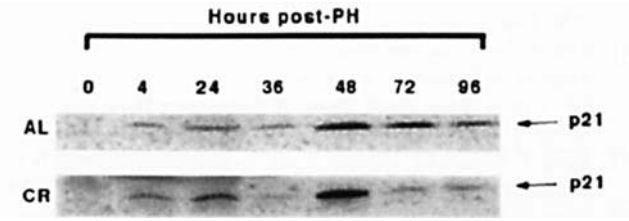

Figure 6. Western blot analysis of p21 protein expression in livers of representative individual AL or CR mice. p21 protein was detected as early as the 4th postoperative hour, and expression continued through the 96th hr, at each interval of assessment in all samples from mice of either dietary group. Peak expression occurred coincident with the second proliferative wave at 48 hr post-PH.

40th postoperative hour intervals. Immediately preceding the second wave of hepatocellular proliferation at 48 hr post-PH, a second peak of E2F-1 protein expression was detected 4 hr earlier in CR mice, at 44 hr compared with 48–52 hr post-PH in AL mice, and was significantly greater among CR mice ($P < 0.01$; Figs. 2 and 3).

The hypophosphorylated form of Rb p110 was detected in livers of either AL or CR mice prior to surgical PH, which is consistent with the expression of adult hepatocytes in the G0 phase (Fig. 4). PH caused the hyperphosphorylated form of Rb p110 to form primarily at the 28-, 36-, and 48-hr intervals after PH in CR mice, and at the 36- and 48-hr intervals in AL mice, coincident with the rise in hepatic DNA-LI described above. During the earliest proliferative wave 28–36 hr after surgical resection, 89% ($n = 9$) of the regenerating livers from CR mice expressed the hyperphosphorylated form of Rb p110 compared with 50% of livers from AL mice, indicating that more hepatocytes were entering into S phase earlier in CR mice. By 96 hr after PH, the hyperphosphorylated form of Rb p110 was rarely and only faintly detectable.

Cyclin A expression was not detected in any liver sample from either cohort prior to 32 hr after partial surgical resection. Cyclin A expression was induced 4 hr earlier in CR mice at 32 hr post-PH, coincident with the first proliferative wave, and was maximally expressed during the second proliferative wave at the 48th postoperative hour in each dietary group (Fig. 5). Peak cyclin A expression extended through the 52nd postoperative hour in AL mice, but subsided sharply in CR mice after the 48th-hr interval. Cyclin A protein was not detected in liver samples of CR mice 72 or 96 hr after PH ($n = 6$), but was expressed in 50% of AL samples, indicating a more acute post-replicative exiting from the cell cycle by hepatocytes in CR mice.

In the quiescent liver prior to partial surgical resection, no p21 protein was detected in any sample from either dietary group. PH induced p21 expression so that it was detected as early as the 4th hr and through the 96th postoperative hour, at each interval of assessment in all samples from mice of either dietary group (Fig. 6). Expression of p21 peaked during the second proliferative wave, and extended into the post-replicative phases of hepatic regeneration 72 and 96 hr post-PH. Although the regenerating livers of some individual CR mice expressed less p21 during the 72nd and 96th hr post-replicative intervals (Fig. 6), mean p21 protein expressions were not significantly attenuated earlier in the CR cohort compared with AL controls.

Discussion

Mitogenic stimulation and the responsiveness of cells may be qualitatively different in CR animals, and may be mediated by adaptive changes in neuroendocrine secretions that serve to maintain plasma glucose levels. CR reduces plasma levels of insulin-like growth factor-1 (IGF-1), and acutely lowers the amplitude of growth hormone secretory pulses in the young animal, which may be sufficient to diminish the general mitogenic stimulation of parenchymal and stromal cells and lower the DNA-LI of tissues *in vivo* (48, 49), including the liver (50–52). But CR also increases IGF-1 receptor density, maintains the paracrine influence and parenchymal levels of IGF-1, and increases both the number and amplitude of growth hormone secretory pulses in the aged CR animal, which may account for the more youthful DNA-LI of tissues and the greater *in vitro* replicative capacity of cells derived from aged CR animals (48, 49).

To test whether CR enhances cell responsiveness, compensatory hyperplasia of the liver following its partial resection was used to evaluate the expressions of cell cycle regulatory proteins during controlled cell proliferation *in vivo*. Compared to *ad libitum*-fed mice, CR mice that consumed 34% fewer calories for 10 weeks demonstrated evidence of accelerated entrance and passage of hepatocytes

through the G1 and S phases of the cell cycle, and an abrupt, earlier exit from regenerative hyperplasia. Regenerating livers of CR mice showed earlier evidence of BrdU-labeled DNA synthesis, earlier and greater expression of the E2F-1 protein, earlier expression of the cyclin A protein, and more CR mice consistently expressed the hyperphosphorylated form of Rb p110 during the first proliferative wave than did AL controls, indicating that more hepatocytes were entering into S phase earlier in CR mice. Peak cyclin A expression during the second proliferative wave continued 4 hr longer in the regenerating livers of AL mice, demonstrating that numerous hepatocytes were still in S phase in AL mice at the 52nd-hr interval, and that hepatocytes of CR mice had exited earlier from the cell cycle to a post-replicative state.

These observations represent the first evidence that CR influences the expression of cell cycle regulatory proteins, and they indicate that hepatocytes in CR mice respond acutely to mitogenic stimuli and efficiently regenerate lost hepatic parenchyma. These findings substantiate our earlier report and the observations of others that CR increases the ^{3}H-thymidine pulse labeling of hepatocellular DNA after PH measured *in vivo* and in cultured hepatocytes *in vitro* (47, 53, 54). Quiescent hepatocytes in the intact liver may be "primed" by CR to respond more acutely to mitogenic stimuli, perhaps by earlier induced peak levels of D-type cyclin/CDKs that would hyperphosphorylate Rb, cause the observed early release of the E2F-1 transcription factor, trigger cell cycle progression, and result in the observed early exit to a post-replicative state with full reconstitution of liver mass, but this has not been determined. In contrast, CR may lower constitutive levels of D-type cyclins in hepatocytes in the intact liver and in other adult cell populations, contributing to reduction in Rb phosphorylation and reduced tissue DNA-LI, but this has not been determined. Overexpression of D-type cyclins has been postulated to lead to over phosphorylation of the Rb protein and to contribute to cell cycle deregulation and carcinogenesis (45, 46). Reduced constitutive expression and greater or earlier mitogen-induced peak expressions of other G1 cyclins, including cyclins C, D, and E, their associated CDKs, or any of the immediate early genes, including *c-fos*, *c-jun*, and *c-myc*, followed by p53 and *c-Ha-ras* that are typically activated within the first day following PH (55, 56), may also contribute to a reduced hepatic DNA-LI in the quiescent liver, and a primed state of hepatocellular responsiveness, but this awaits determination. In addition, CR may increase hepatocellular activation by increasing the receptor density, circulating level, or paracrine activities of mitogenic factors.

Cells must integrate and respond to a plethora of signals before choosing to commit to S phase, and the rate, manner, and fidelity with which they respond and progress through the cell cycle may influence risk for cancer and aging disorders. Translation of the beneficial consequences of CR to the prevention of cancer and delay of aging in humans awaits the definition of the protective mechanism, and may require the development of a surrogate intervention with a comparable outcome.

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