MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 6917–6925 Vol. 16, No. 12
0270-7306/96/$04.00+0

Convergence of Mitogenic Signalling Cascades from Diverse Classes of Receptors at the Cyclin D–Cyclin-Dependent Kinase–pRb-Controlled G_1 Checkpoint

JIRI LUKAS, JIRINA BARTKOVA, AND JIRI BARTEK*

Division of Cancer Biology, Danish Cancer Society, DK-2100 Copenhagen Ø, Denmark

Received 29 July 1996/Returned for modification 8 September 1996/Accepted 18 September 1996

The commitment of mammalian cells in late G_1 to replicate the genome and divide in response to mitogenic growth factors operating via tyrosine kinase receptors depends on phosphorylation of the retinoblastoma protein (pRb), a process controlled by cyclin D-associated cyclin-dependent kinases (cdks) and their inhibitors. This study addressed the issue of whether also other mitogenic signalling cascades require activation of cyclin D-associated kinases or whether any mitogenic pathway can bypass the cyclin D-pRb checkpoint. We show that mitogenic signal transduction pathways from three classes of receptors, the membrane tyrosine kinase receptors activated by serum mitogens or epidermal growth factor, estrogen receptors triggered by estradiol, and the cyclic AMP-dependent signalling from G-protein-coupled thyrotropin receptors, all converge and strictly require the cyclin D-cdk activity to induce S phase in human MCF-7 cells and/or primary dog thyrocytes. Combined microinjection and biochemical approaches showed that whereas these three mitogenic cascades are sensitive to the p16 inhibitor of cdk4/6 and/or cyclin D1-neutralizing antibody and able to induce pRb kinase activity, their upstream biochemical routes are distinct as demonstrated by their differential sensitivity to lovastatin and requirements for mitogen-activated protein kinases whose sustained activation is seen only in the growth factor-dependent pathway. Taken together, these results support the candidacy of the cyclin D-cdk-pRb interplay for the convergence step of multiple signalling cascades and a mechanism contributing to the restriction point switch.

The fundamental decision of mammalian cells as to whether to replicate their DNA and divide or to withdraw from the cell cycle with an unduplicated genome takes place in mid- to late G_1 phase. This critical G_1 checkpoint, often referred to as the restriction (R) point (51), operationally separates the cell cycle into an initial mitogen-dependent period and a largely cell-autonomous, growth factor-independent period from the commitment to enter S phase until cell division (4, 51, 62, 63, 66, 73). The commitment process at the G_1 checkpoint reflects complex integration of a plethora of positive and negative extracellular and intracellular signals transduced by multiple cascades into the cell nucleus (19, 21, 41, 61). As these signal transduction pathways must ultimately pass their stimuli on the cell cycle machinery itself, understanding the molecular basis of the link between the upstream signalling circuitry and the cell cycle clock is of immense interest for biomedicine (4, 18, 61, 63). The significance of elucidating the regulation at the R point is further underlined by its involvement in biological processes as diverse as cell proliferation, temporary cell cycle arrest to allow for DNA repair, quiescence state in response to growth factor deprivation, terminal differentiation, senescence, or cell death (4, 18, 51, 63, 66, 67). In addition, any malfunction of the G_1 checkpoint may result in unrestricted cell proliferation and/or genetic instability, possibly leading to development of cancer or other diseases (4, 17, 18, 23, 51, 63, 66).

Although the precise biochemical nature of the commitment transition remains to be clarified, recent discoveries of the mammalian G_1 cyclins, their partner kinases, inhibitors, and candidate substrate(s) offer a plausible scenario for the restriction point control. The decision to proceed beyond the late G_1 appears to be executed by phosphorylating and thereby inactivating the product of the retinoblastoma gene (pRb), whose growth-restraining role in early to mid-G1 reflects its ability to sequester a series of transcription factors the activities of which are required to induce expression of S-phase genes (4, 16, 50, 66). The critical phosphorylation of pRb in mid to late G1 is most likely carried out by the D-type cyclins (cyclins D1, D2, and/or D3) and their partner cyclin-dependent kinases (cdks) cdk4 and cdk6 (4, 6, 34, 42, 46, 56, 62, 63, 66). The D-type cyclins are induced as part of the delayed early response to mitogenic stimulation by growth factors, form active holoenzymes with cdk4 or cdk6 by mid-G_1, are able to bind directly to pRb via their N-terminal L-X-C-X-E motifs, have a substrate preference for pRb over histone H1, and phosphorylate pRb in vitro on residues which are physiologically phosphorylated in G_1 in vivo (4, 60, 62, 66). Consistent with a major role in positive regulation of G_1 progression, the D-type cyclins are required for S-phase entry, and their overexpression accelerates G_1 and reduces dependency on exogenous growth factors (3, 4, 5, 35, 36, 37, 49, 54, 55). In addition, the inhibition of cyclin D1 or cdk4/6 by neutralizing antibodies or overexpression of $p16^{INK4a}$, a cdk inhibitor specific for cdk4 and cdk6 (59), prevents progression into S phase only in cells harboring functional pRb, suggesting that pRb is the major substrate of cyclin D-dependent kinases (28, 34, 36, 38, 44, 64). Although cyclin E is also rate limiting for G_1 progression and may contribute to phosphorylation of pRb, its peak abundance and associated cdk2 kinase activity are delayed relative to D cyclins and it appears to function at the G_1/S transition by phosphorylating some so far unknown substrate(s) other than pRb (22, 46, 53, 56, 62). These data point to cyclin D-associated kinases and their pRb substrate as the most plausible candidates for controlling the critical mammalian checkpoint in advanced G_1, a notion further supported by frequent oncogenic aberrations

* Corresponding author. Mailing address: Danish Cancer Society, Division of Cancer Biology, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark. Phone: 45-35 25 75 00. Fax: 45 31 38 20 44.

of individual components of this pathway (4, 17, 18, 23, 26, 33, 50, 63, 66).

One important prediction from the candidacy of the cyclin D-pRb interplay for the R-point switch is that it would be targeted by multiple mitogenic signalling cascades, including those transduced from non-tyrosine kinase receptors, for which an essential role of cyclin D-cdks in commitment to DNA synthesis has not been demonstrated so far. This issue clearly merits experimental clarification, since the pRb-dependent checkpoint is apparently not required for the early mammalian embryonic cell cycles (8, 24, 31), and it remains to be seen whether any of the mitogenic signalling cascades which normally control proliferation of pRb-positive cells could stimulate entry into S phase through a mechanism bypassing the requirement for pRb phosphorylation by cyclin D-dependent kinases. On the other hand, if all major mitogenic pathways require passage through the cyclin D-pRb switch, an important task would be to pinpoint the level(s) at which the diverse cascades converge to trigger the activity of cyclin D-dependent kinases and pRb phosphorylation. We have addressed some of these issues experimentally and demonstrate here that (i) the mitogenic stimuli from three distinct signalling cascades, namely, those activated by growth factors via tyrosine kinase receptors, by estradiol via estrogen receptors, and by cyclic Amp (cAMP)-elevating agents via G-protein-coupled thyrotropin receptors, all converge and strictly require the cyclin D-cdk activity to promote S-phase onset; and (ii) the upstream biochemical modes (including the requirement for mitogen-activated protein kinase [MAPK] activity) of the cyclin D-cdk holoenzyme activation differ significantly among the three mitogenic cascades examined, suggesting that these pathways converge downstream of the MAPK-dependent step.

MATERIALS AND METHODS

Cell culture and synchronization. Human breast cancer cell lines MCF-7, BT-549, and T-47D and the canine MDCK cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 5 or 10% fetal calf serum, 2 mM glutamine, penicillin (10 U/ml), and streptomycin (10 U/ml). The T-47D cell line with zinc-inducible cyclin D1 expression (49) was a kind gift of Elizabeth Musgrove. Primary dog thyroid epithelium was isolated and cultured exactly as reported by Lamy et al. (29), and cells synchronized by growth factor starvation were stimulated by either thyroid-stimulating hormone (TSH; 1 mU/ml; Sigma), forskolin (20 μM; Sigma), epidermal growth factor (EGF; 100 ng/ml; Sigma), or a combination of TSH or forskolin with EGF (29). The experiments with MCF-7 cells were initiated by 3-day culture in phenol red-free medium with 5% charcoal-stripped fetal calf serum, followed by addition of lovastatin (20 μM; a kind gift from Merck, Sharp & Dohme Laboratories) for 30 to 36 h (27) and subsequent stimulation with either removal of lovastatin and addition of mevalonate (2 mM; Sigma) plus serum or mevalonate plus EGF (5 ng/ml) and insulin (100 ng/ml; Sigma) or stimulation with 17β-estradiol (E2; 50 nM; Sigma) in the presence of fresh lovastatin (see also reference 7). The steroidal antiestrogen ICI 182,780, used at 5 μM in some control experiments, was kindly provided by Anne Lykkesfeldt.

Antibodies and plasmids. Mouse monoclonal antibodies DCS-6, DCS-11 (34, 37), and 5D4 (donated by M. Sato) to cyclin D1, monoclonal antibody 245 to pRb (donated by W.-H. Lee), and DCS-50 to p16 (38) were used as hybridoma tissue culture supernatants or immunoglobulin G (IgG) affinity purified on protein A. Rabbit antisera to cdk4 (C-22), ERK-1 (C-16), and ERK-2 (C-14) kinases were purchased from Santa Cruz, and the mouse antibody against the CD20 membrane marker was from Becton Dickinson. The p16 wild-type cDNA in pGEX-2TK (Pharmacia) was used in *Escherichia coli* BL21(DE3)pLysS to express the glutathione *S*-transferase (GST)–p16 fusion protein and as pX-myc/p16 vector in mammalian cells (38). The expression plasmids pCMV-CD20 and pEXV3 with either wild-type or dominant-negative (S221A) mutant MAPKK were donated by Kristian Helin and Chris Marshall, respectively.

Cell cycle analyses. Progression of cells through the cell cycle was monitored either by flow cytometry analysis of DNA content of cell populations stained with propidium iodide (35) or by immunofluorescence detection of bromodeoxyuridine (BrdU) incorporation in cells cultured and fixed in situ (38) at various time points as indicated in the text. The BrdU labeling period covered entire duration of the experiments with human T-47D cells; the drug was added to the medium immediately after microinjections into MCF-7 or the dog thyroid cells and therefore was present for at least the last 24 h before evaluation.

Immunochemical analyses. Extraction of native proteins from exponential cell cultures, estimation of protein concentration, and immunoprecipitation protocols were described previously (34, 37). For cdk4-p16 complex formation in vitro, cell lysates (1 mg) were incubated with soluble GST-p16 fusion protein (1 μg) for 1 h on ice, followed by collection of the formed complexes on glutathione-Sepharose 4B (Pharmacia) and immunoblot analysis with antiserum to cdk4. Equal amounts of extracted or immunoprecipitated proteins were electrophoretically separated on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and blotted onto nitrocellulose by a semidry method, and immunodetection was performed with appropriate primary antibodies and an enhanced chemiluminescence system (Amersham, Aylesbury, England) according to the manufacturer's instructions. Immunofluorescence detection of either BrdU incorporation or expression of cellular or microinjected proteins was performed as described previously (36), using a combination of Texas red-conjugated and fluorescein isothiocyanate-conjugated secondary reagents and Hoechst to counterstain DNA.

Gene and protein microinjection. Cells synchronized with lovastatin (MCF-7) or by growth factor starvation (primary dog thyrocytes) were microinjected with either the affinity-purified GST-p16 wild-type fusion protein (2 mg/ml), affinity-purified monoclonal antibody DCS-6 to cyclin D1 (6 mg/ml), or control mouse immunoglobulin as described previously (38), using a Zeiss AIS microinjection apparatus. Coinjections of p16 (25 μg/ml) and CD20 (5 μg/ml) expression plasmid DNA into synchronized cells were done as described previously (39). Cell cycle progression of the stimulated cells was evaluated by combined in situ double-immunofluorescence analysis of BrdU incorporation and p16, CD20, or immunoglobulin contents as described previously (34, 38, 39). Each experiment was performed two to four times to verify reproducibility.

Kinase assays. MAPK activity was measured in vitro after immunoprecipitation of p42 ERK-1 (antibody C-14; Santa Cruz) and p44 ERK-1 (antibody C-16; Santa Cruz), using myelin basic protein (MBP; Sigma) as a substrate. Cells were lysed on ice in lysis buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 1 mM Na_3 VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, and 2 μg of aprotinin per ml, and the protein extracts were clarified by centrifugation. For each immunoprecipitation, 100 μg of total protein was incubated for 1 h at 4°C with 10 μl of protein A beads (Pharmacia) saturated with anti-ERK or control rabbit preimmune immunoglobulins. After extensive washing in lysis buffer, the beads were assayed for 15 min at 30°C in 30 μl of kinase assay buffer (20 mM HEPES [pH 8.0], 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mm dithiothreitol, 5 μCi of [γ-^{32}P]ATP, 20 μM nonlabeled ATP) containing 0.25 mg of MBP per ml. The cyclin D1-associated kinase activity toward the GST-pRB large pocket fusion protein as the substrate (42) was measured essentially as described previously (34) except for the concentration of the nonlabeled ATP, which was increased to 50 μM in this study. Kinase reactions were analyzed by SDS-polyacrylamide gel electrophoresis and quantitated by a PhosphorImager (Molecular Dynamics).

RESULTS

Estrogen-induced and growth factor-induced mitogenesis in MCF-7 cells requires functional cyclin D-dependent kinases. To examine whether the cyclin D-dependent kinase activity is essential for estrogen-induced commitment to enter S phase, we used the human estrogen-responsive MCF-7 breast cancer cell line synchronized in early G_1 by lovastatin (7, 27), an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase; EC 1.1.1.88), whose activity in the cholesterol biosynthesis pathway converts HMG-CoA to mevalonate (40). The G_1-inhibitory effect of lovastatin is not fully understood but appears to be caused, at least in part, by preventing isoprenylation, a modification required for membrane anchorage and function, of a set of signal transduction mediators including $p21^{ras}$ and related G proteins such as Rho (11, 20, 25, 30, 40). In contrast to its proliferation-inhibitory properties on cells growing in response to serum or polypeptide growth factors (11, 25, 27, 40), lovastatin and simvastatin (another inhibitor of HMG-CoA reductase) have recently been shown to be without detectable effect on cell cycle progression of MCF-7 cells mitogenically stimulated by E2 (7), thus opening the way to study the latter under conditions of a selective blockade of the former pathway. We have previously shown that MCF-7 cells stimulated by serum growth factors require functional cyclin D1 to pass the G_1 checkpoint (5, 36) and that the D-type cyclins and their partner kinases operate upstream of pRb within the same G_1-regulatory pathway, most likely by

directly phosphorylating pRb in mid to late G_1 (34, 36, 38). In this study, subconfluent MCF-7 cells were synchronized in early G_1 by 35-h exposure to 20 μM lovastatin under estrogen-free conditions (see Materials and Methods), followed by one of the three mitogenic protocols: (i) stimulation with E2 in the presence of fresh lovastatin, (ii) removal of lovastatin and addition of fresh medium supplemented with serum and mevalonate, or (iii) removal of lovastatin and addition of serum-free medium supplemented with mevalonate, EGF, and insulin. While the lovastatin treatment virtually eliminated the S-phase fraction and blocked over 90% of MCF-7 cells in G_1, each of the three mitogenic protocols resulted in stimulation of cell cycle progression, with the cells entering S phase between 12 and 24 h after stimulation. The E2-stimulated cells progressed through G_1 moderately faster, reflected by an approximately 4-h acceleration of S-phase onset compared with either the EGF- or serum-stimulated counterpart cell populations (references 7 and 27 and our unpublished data). Parallel control experiments showed that the proliferation-stimulatory effect of serum or EGF with mevalonate could be totally prevented by continuous exposure to 20 μM lovastatin, whereas E2 stimulation was abolished by addition of the steroidal antiestrogen ICI 182, 780 (5 μM), thereby confirming the specificity of the mitogenic effects (our unpublished data and reference 7).

To examine the requirements for the cyclin D-cdk function, MCF-7 cells stimulated by each of the three protocols were microinjected in early G_1, within 2 to 4 h poststimulation, with either the cyclin D1-neutralizing monoclonal antibody DCS-6 (34, 37) or the GST-p16 fusion protein known to inhibit cyclin D-associated cdk4 or cdk6 (28, 33, 38, 52, 59), and the effects of these treatments upon G_1/S progression were evaluated against the control microinjections of nonspecific immunoglobulin and/or comparison with noninjected cells (Fig. 1a to f and 2). In a complementary set of experiments, MCF-7 cells were microinjected, at the time of stimulation, with an expression plasmid encoding wild-type human p16, and the effects were compared with those of control DNA (Fig. 1g and h and 2). Monitoring S-phase entry by in situ immunofluorescence analysis of BrdU incorporation (38) showed that MCF-7 cells mitogenically stimulated by any of the three growth-promoting protocols were inhibited in G_1 either by neutralization of cyclin D1 or via excess p16 (both in the form of the GST-p16 fusion protein or when expressed from the microinjected plasmid) (Fig. 1 and 2). In conclusion, these results demonstrate that the activity of cyclin D-associated cdks is essential for progression through G_1 in MCF-7 cells, whose proliferation is driven either by polypeptide growth factors acting via the respective membrane tyrosine kinase receptors or by estradiol acting via the nuclear estrogen receptors.

Distinct requirements for MAPK activation and induction of cyclin D/cdk as pRb kinase by different mitogens. The microinjection experiments performed with MCF-7 cells stimulated to proliferate by different mitogens showed that the activity of cyclin D-associated cdks is required for S-phase entry in either pathway, but this approach could not reveal the dynamics or biochemical basis of the cyclin D-cdk activation by the distinct signalling cascades. Since the critical biochemical parameter of the cyclin D-cdk function is its kinase activity, we examined the dynamics of the cyclin D1-associated pRb kinase activity after mitogenic stimulation of MCF-7 cells by either E2 or EGF. A preliminary assessment of the specificity of the three mouse monoclonal antibodies to cyclin D1 in this assay, and modification of the reaction conditions to eliminate any background signal, was carried out, and the result is shown in Fig. 3a. The control DCS-6 antibody recognizing only the cdk-free form of cyclin D1 (34) did not coprecipitate any pRb kinase activity from extracts of exponentially growing MCF-7 or BT-549 cells, whereas both 5D4 and DCS-11 antibodies, known to recognize also the cdk4-bound cyclin D1 (34),

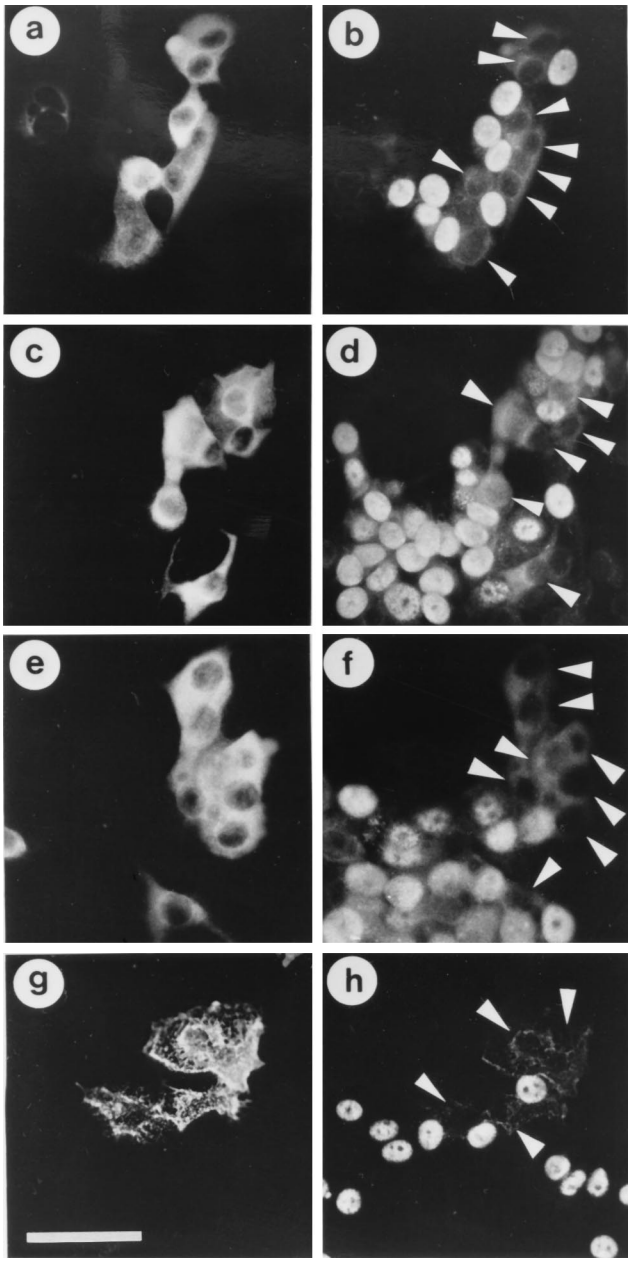

FIG. 1. Microinjections of either cyclin D1-neutralizing antibody (DCS-6) or p16 (purified GST fusion protein or expression plasmid) prevent entry into S phase in MCF-7 cells synchronized by lovastatin and stimulated by different mitogens. (a and b) Cells stimulated with 10% fetal calf serum, removal of lovastatin, and addition of mevalonic acid (2 mM) were microinjected with GST-p16 (2 mg/ml) together with mouse nonimmune IgG (1 mg/ml) as a marker for successfully injected cells. (c and d) Cells stimulated with E2 in the presence of lovastatin, microinjected with GST-p16. (e and f) Stimulation as for panels c and d but the cells injected with DCS-6 (6 mg/ml). (g and h) Cells stimulated with EGF and mevalonic acid and coinjected with p16 expression plasmid and the plasmid encoding the CD20 membrane marker to identify productively injected cells. (a, c, e, and g) Detection of microinjected cells; (b, d, f, and h) the same fields stained for BrdU. BrdU was added immediately after microinjections for continuous labeling. Arrows point to microinjected cells. Scale bar = 25 μm.

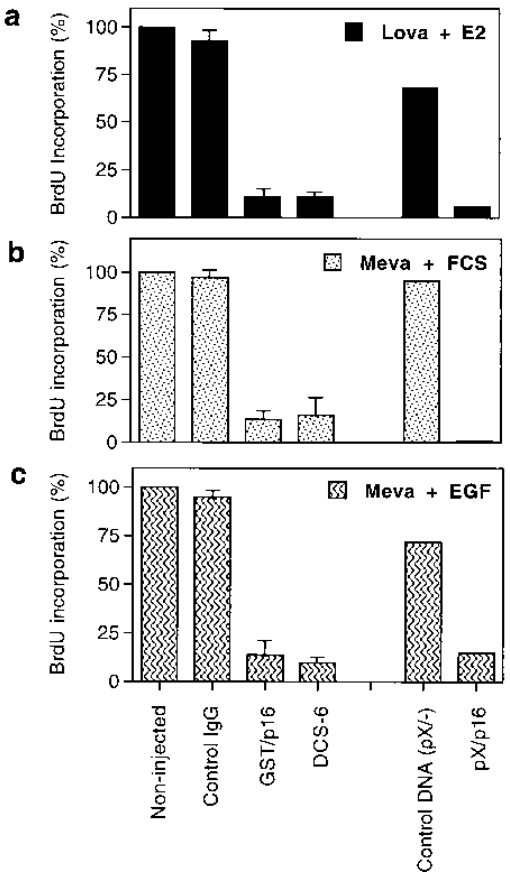

FIG. 2. Graphs summarizing inhibition of E2-, serum-, or EGF-induced mitogenesis in MCF-7 cells by cyclin D1-neutralizing antibody (DCS-6), GST-p16 protein, or p16 expression plasmid. Cells were microinjected with various reagents (as indicated at the bottom) prior to stimulation with E2 in the presence of lovastatin (Lova) (a), mevalonic acid (Meva) and 10% fetal calf serum (FCS) (b), or mevalonic acid and EGF (c), and their progression into S phase was assessed 30 h poststimulation by BrdU incorporation (BrdU was present for the last 24 h before evaluation). The graphs represent mean values from four independent experiments ± standard deviations for control IgG, GST-p16, and DCS-6 microinjections and examples of one of two experiments with p16-encoding or control vector injections.

brought down active complexes from extracts of MCF-7 but not of BT-549 cells (Fig. 3a), the latter being a control Rb-deficient breast cancer cell line expressing a high level of the p16 inhibitor and no detectable cyclin D1-cdk complexes (33, 52). The pRb kinase activities measured in MCF-7 cell extracts at 4-h intervals between 0 and 20 h of mitogenic stimulation are illustrated in Fig. 3b. The cyclin D1-associated kinase activity rose faster in the estradiol-stimulated cells, reaching a plateau level at 12 h, while in the EGF-stimulated cultures, the pRb kinase peaked slightly later, at 16 h of mitogenic treatment (Fig. 3b). The dynamics of cyclin D-cdk activity correlated well with the appearance of hyperphosphorylated endogenous pRb form detected by immunoblotting (data not shown), and the approximately 4-h acceleration upon stimulation with estradiol compared with EGF or serum was highly reproducible in four independent experiments.

The differential sensitivity toward lovastatin and the moderately different dynamics of cyclin D1-dependent kinase activation between the signalling cascades initiated from estrogen receptors versus membrane tyrosine kinase receptors indicated that their upstream routes, which eventually activate the cyclin D-cdk holoenzymes, may be distinct. A convenient signal transducing step at which to verify this possibility appeared to be the MAPK involvement in either pathway. As expected, the ERK-2-associated kinase activity toward the MBP substrate showed a biphasic curve in extracts of MCF-7 cells stimulated by EGF (ERK-1 showed similar dynamics but lower overall activity). An initial increase of the activity detected at 10 min after stimulation was followed by a partial decrease and then by a sustained elevated activity for up to 6 h after EGF addition (Fig. 3c). In contrast, parallel examination of cells stimulated by E2 showed a different activity pattern whose lower and transient initial peak was followed by a rapid decline from which the kinase activity did not recover for the duration of the experiment (Fig. 3c). Since mitogenic effects, as tested in various model systems, require sustained activation of MAPKs for several hours (45, 72), our results indicated that the MAPK pathway is not required for the estradiol-induced commitment to DNA synthesis in MCF-7 cells. This interpretation is also consistent with our finding that microinjection of an expression plasmid (at 100 μg/ml) encoding the dominant-negative mutant of MAPKK capable of significantly reducing the MAPK activation (10) did not appreciably interfere with the estradiol-induced cell cycle progression in MCF-7 cells. Thus, whereas the specific inhibition of S-phase entry mediated by the dominant-negative mutant MAPKK was around 40% in cells stimulated by EGF, the effect of the mutant was marginal if any, the inhibition never exceeding 10%, in parallel experiments with E2-stimulated cells.

Taken together, our data demonstrated that the activity of D-cyclin-associated cdks is essential for S-phase entry induced in MCF-7 cells by either growth factor or estradiol treatment and that the two pathways are biochemically distinct. One prediction from these results would be that a selective elevation of cyclin D-cdk complex in the absence of any mitogen might be able to drive the cells into DNA synthesis. To perform such an experiment without the otherwise interfering autocrine growth factors known to be produced by the estrogen-responsive breast cancer cell lines, we took advantage of the zinc-inducible cyclin D1 expression system in the human T-47D breast cancer cell line (49) combined with lovastatin treatment. Titration experiments with increasing levels of zinc sulfate under serum-free, lovastatin-free conditions identified the 30 to 50 μM concentration as optimal for induction of cyclin D1 expression and its mitogenic effects (Fig. 4a and reference 49). In medium free from exogenous mitogens, the T-47D cells show a small fraction of S-phase cells, which can be effectively eliminated by addition of 20 μM lovastatin (Fig. 4b). As expected, addition of 2 mM mevalonate reverses the inhibitory effect of lovastatin, while addition of 10% serum in the presence of lovastatin has little effect. The critical experiment was then performed by adding the increasing concentrations of zinc sulfate to T-47D cells in mitogen-free medium in the presence of lovastatin, resulting in a dose-dependent increase of S-phase cells. This result confirmed that even in the absence of estrogen, and under conditions such that the tyrosine kinase receptor-Ras-MAPK pathway is blocked by lovastatin, elevated cyclin D1 is sufficient to overcome the lack of mitogenic stimuli and trigger progression into S phase.

Both cAMP-stimulated signalling and EGF-stimulated mitogenic signalling in thyroid epithelium are cyclin D-cdk dependent. The convergence on the cyclin D-cdk-pRb-dependent checkpoint of the mitogenic cascades initiated from two classes of receptors in MCF-7 cells raised the question of whether other proliferative stimuli share the ability to target cyclin D-associated cdks. To test this possibility, we used the well-defined model of dog thyroid epithelium, in which the mito-

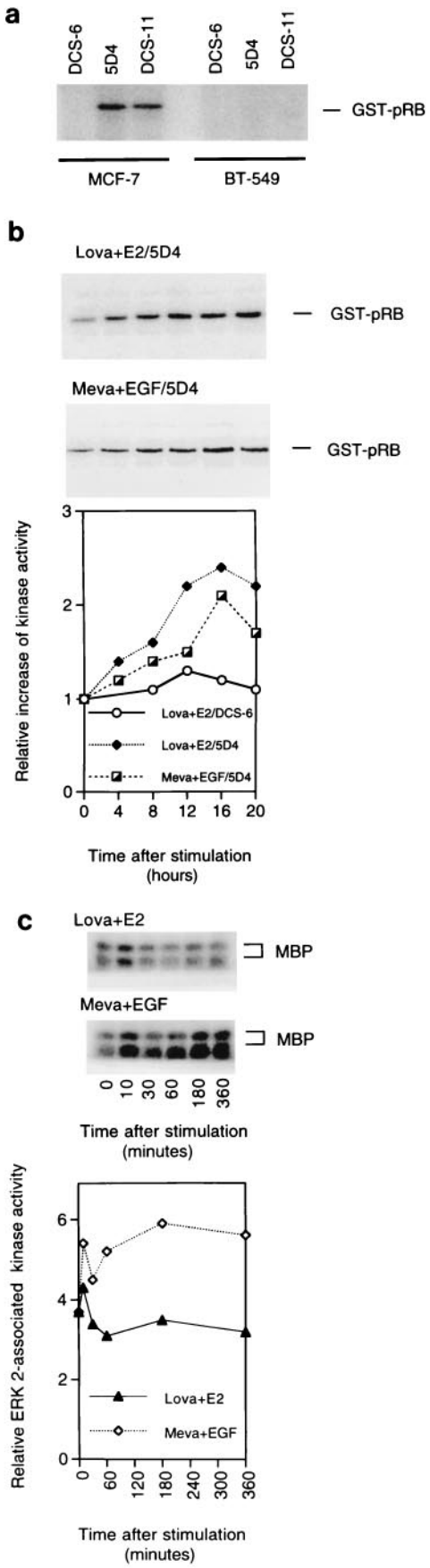

FIG. 3. Effects of stimulating different mitogenic pathways on cyclin D-dependent pRb kinase and MAPK activity in MCF-7 cells. (a) Determination of the specificity of anti-cyclin D1 monoclonal antibodies (indicated on the top) to support in vitro pRb kinase activity immunoprecipitated from MCF-7 or BT-549 cell extracts. (b) Dynamics of cyclin D1-associated pRb kinase activity (antibody 5D4) upon E2 (upper panel) or EGF (middle panel) stimulation. The bottom panel shows mean values from phosphor image quantitation of several independent experiments performed with different anti-cyclin D1 antibodies, including DCS-6 as a negative control. Lova, lovastatin; Meva, mevalonic acid (c) Differential activation of ERK-2 kinase via different mitogenic stimuli as indicated, measured by MBP as a substrate. The upper two panels show representative examples of kinase reactions; the lower panel presents their phosphor image quantitation. Note the absence of the sustained ERK-2 activation after stimulation with E2.

genic pathways stimulated either by polypeptide growth factors such as EGF or by cAMP-elevating agents such as the TSH (13, 29) can be studied separately. These two cascades are initiated from diverse classes of receptors, and their distinct biochemical nature is evident from the fact that the tyrosine kinase receptor-mediated, but not the cAMP-mediated, mitogenesis requires activation of the MAPK in these cells (29).

Cultured primary dog thyroid epithelial cells were synchronized by starvation for 4 days in serum-free medium, followed by stimulation of parallel cultures with various mitogens, and the effect of the GST-p16 fusion protein microinjected in early G1 upon G_1/S progression was examined by in situ monitoring of BrdU incorporation. Microinjection of the GST-p16 protein, but not of the control protein, prevented S-phase entry of

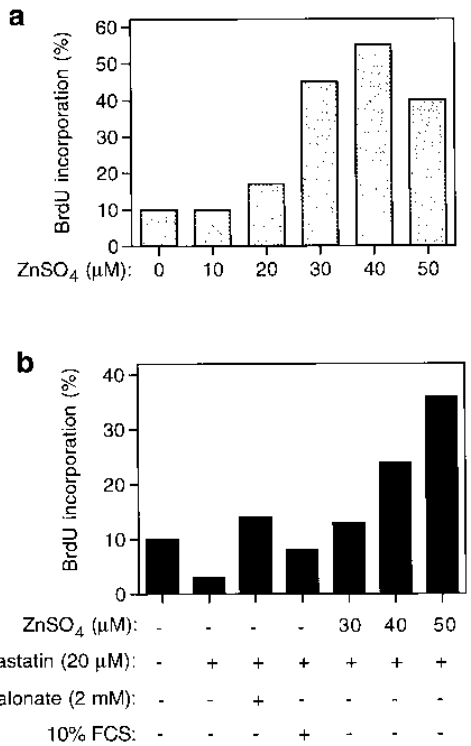

FIG. 4. Cyclin D1 activation is sufficient to overcome the lovastatin-mediated cell cycle arrest in the absence of mitogens. (a) S-phase induction in growth factor-starved T-47D cells with a zinc-inducible cyclin D1 transgene treated for 12 h with the indicated concentrations of zinc sulfate; (b) S-phase fractions of T-47D cells presynchronized with 20 μM lovastatin for 36 h, followed by 22 h of differential treatment as indicated at the bottom. BrdU was continuously present from the time of stimulation, and its incorporation was evaluated by in situ immunofluorescence. FCS, fetal calf serum.

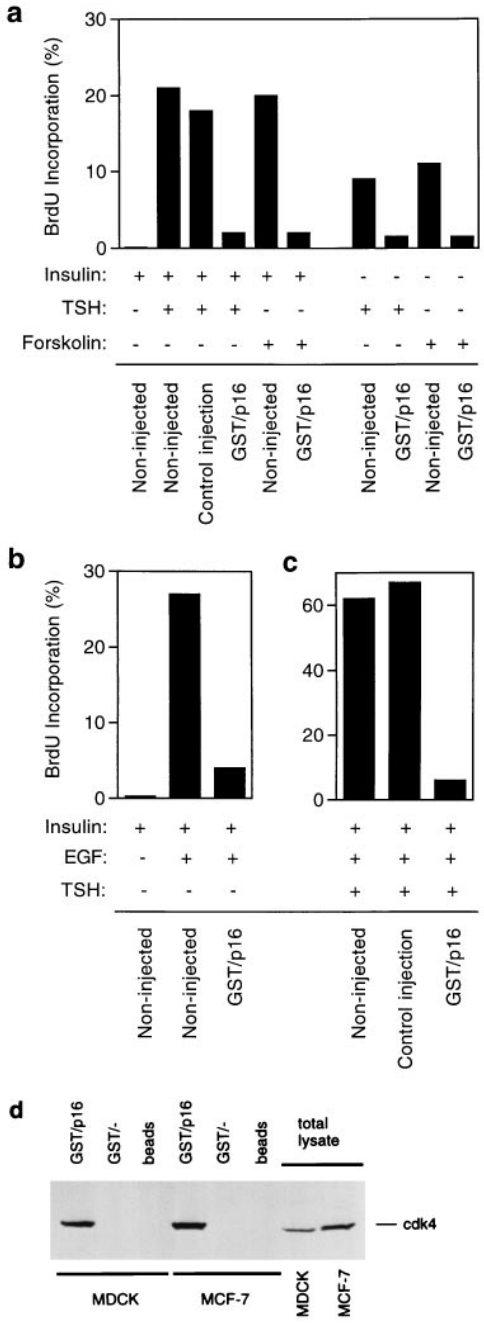

FIG. 5. Summary of microinjection experiments with dog thyrocytes stimulated to proliferate via different mitogenic pathways. Cells microinjected with purified GST-p16 fusion protein or control IgG were stimulated to proliferate by TSH or forskolin (a), by EGF (b), or by combined treatment of TSH plus EGF (c), with or without insulin as indicated. After 36 h, BrdU incorporation was evaluated by in situ immunofluorescence (BrdU was present for the last 30 h before evaluation). Shown are cumulative data from independent experiments with cells isolated from two animals. (d) Human GST-p16 fusion protein used in all microinjection experiments is able to quantitatively associate with cdk4 of human and canine origin. Protein lysates of human MCF-7 or canine kidney cell line MDCK were incubated with immobilized GST-p16, free GST, or plain glutathione-Sepharose beads. The bound complexes were released by boiling and analyzed by immunoblotting for the presence of cdk4.

the thyroid epithelium stimulated to proliferate by TSH or forskolin (Fig. 5a), a cAMP-elevating agent operating through the same pathway as TSH (13, 29). As expected, the GST-p16 protein inhibited G_1 progression also in parallel cultures stimulated by EGF (Fig. 5b) and even those costimulated by the mitogenically much more potent mixture of EGF and TSH (Fig. 5c). Our finding of only partial mitogenic effects of either EGF or TSH-forskolin (either alone or in combination with nonmitogenic levels of insulin) and their additive effects upon proliferation of the primary dog thyroid epithelium (compare the BrdU incorporation rates of the noninjected cells in Fig. 5a to c) is consistent with previous reports on this model system (13, 29). Finally, confirming that the human p16 protein used in these experiments was able to specifically bind the canine homologue of cdk4, the GST-p16 fusion protein was able to specifically pull down comparable amounts of cdk4 from extracts prepared from either the exponentially growing dog epithelial cell line MDCK or control human MCF-7 cells (Fig. 5d).

We conclude from these experiments that also the cAMP-stimulated mitogenic cascade eventually converges with other pathways to positively regulate the activity of cyclin D-associated cdks required for G_1 progression.

DISCUSSION

The principal novel data in this study demonstrate convergence of distinct signalling cascades initiated from three classes of receptors on cyclin D-cdk, thereby significantly strengthening the proposed candidacy of the cyclin D-cdk-pRb-controlled checkpoint as a potential sensor and integrator of diverse mitogenic stimuli with the cell cycle machinery. This hypothesis (60, 61) evolved based on earlier evidence for a G_1-regulatory function of pRb (16, 66, 67) and on more recent findings that link the D-type cyclins with growth factor-triggered mitogenesis (61–63, 66). In fact, one way cyclin D1 gene was cloned was due to its participation in the delayed early response upon mitogenic stimulation of macrophages by colony-stimulating factor 1 (43). In the macrophage model, colony-stimulating factor 1 is required for continuous expression of cyclin D1 (43), whose overexpression in turn contracts G_1 and renders the cells less sensitive to growth factor deprivation (49, 54, 55). Induction of cyclin D expression is also part of the mitogenic responses of various mesenchymal or epithelial cells to serum or defined growth factors such as platelet-derived growth factor, EGF or insulin-like growth factor I (15, 48, 69, 71) and in T lymphocytes stimulated by phytohemagglutinin and interleukin 2 (1). All of these mitogens operate via signalling cascades involving tyrosine kinase receptors and G proteins known to transmit the signals, at least in part, through the Ras-Raf-MAPK pathway (19, 21, 41). The notion of D-type cyclins as downstream effectors of this pathway is also confirmed by induction of cyclin D1 expression and G_1 acceleration by activated *ras* or *raf* oncogenes themselves (32, 68), and particularly by demonstrations that the cyclin D-cdk function is required for G_1 progression in response to serum growth factors or Ras (3, 37, 54, 58).

Whereas the functional link between the tyrosine kinase receptor pathways and cyclin D-associated cdks appears well established, little is known about the G_1-phase effectors of mitogenic signalling cascades triggered by other classes of receptors. For instance, no data are available on the role of D-type cyclins in cAMP-stimulated mitogenesis initiated in some cells via triggering the G-protein-coupled receptors such as the thyrotropin (TSH) receptor (13). Similar to the cAMP-induced mitogenesis in Swiss 3T3 cells (70), the cAMP-dependent mitogenic cascade elicited by TSH or forskolin in dog thyroid epithelium does not involve phosphorylation and nuclear translocation of p42 and p44 MAPK at any time during the G_0-to-S phase transition, in contrast to EGF-stimulated

mitogenesis in these cells (29). These biochemical differences show a clear distinction between these two pathways in dog thyrocytes, further supported by the fact that TSH induces both proliferation and differentiation whereas EGF leads to proliferation and loss of differentiation (13). Our present results show that the p16 inhibitor of cdk4/6 blocks G_1 progression induced by either EGF or cAMP-elevating agents, implying that both the EGF-dependent and the cAMP-dependent mitogenic cascades converge on a downstream step whose execution requires the activity of cyclin D-dependent kinases.

Our data also help clarify the presently conflicting views of the molecular events involved in the mitogenic cascade elicited by estrogens. The alternative explanations proposed so far include direct transcriptional regulation via ligand-activated estrogen receptors binding to specific response elements of the target gene promoters, stimulation of autocrine growth factor production eliciting indirect effects via membrane tyrosine kinase receptors, or molecular cross talk between estrogens or their receptors and tyrosine kinase receptor pathways (references 7 and 47 and references therein). Recently, two seemingly opposing reports on the potential involvement of MAPK activation in estrogen-stimulated mitogenesis in MCF-7 cells were published. Migliaccio et al. (47) concluded that estradiol stimulates the Ras-Raf-MAPK pathway with similar kinetics as the polypeptide growth factors operating via the membrane tyrosine kinase receptors, implying that estrogens activate mitogenesis through this cascade. On the other hand, Bonapace et al. (7) found no detectable activation of the MAPKs and concluded that estradiol-induced mitogenesis in MCF-7 cells is totally independent of the Ras-Raf-MAPK pathway. We believe that our present data can reconcile these discrepancies. Our results show that estradiol does activate the MAPK in MCF-7 cells but only very transiently, without the sustained kinase activity which is a prerequisite for their mitogenic effect in other receptor-ligand systems or v-*src*-dependent mitogenesis (45, 72). The initial moderate activation of the MAPK activity was not seen by Bonapace et al. (7) due to a long interval between estrogen addition and the first time point examined, but these authors were right in concluding that the estradiol-driven mitogenesis is largely MAPK independent. Our data are consistent with the notion that polypeptide growth factors and estradiol elicit mitogenic responses in MCF-7 cells via two distinct signalling cascades, the former sensitive to lovastatin and dependent on MAPK activation and the latter bypassing both the lovastatin-sensitive step and requirement for MAPK activity.

Regardless of the upstream components involved, previous results showing increased cyclin D1 expression following progestin treatment (48) and correlation of antiestrogen-induced inhibition of proliferation with decreased activity of cyclin D-associated cdks (65) suggested a possible role for these G_1 regulators in mediating mitogenic action of estrogenic hormones. While this report was in preparation, two groups reported stimulation of cyclin D-associated kinase activity by estrogens, thus independently confirming one of our present experiments (2, 14). On the other hand, some of these effects might have been influenced by the autocrine polypeptide growth factors known to be secreted by the breast cancer cell lines, and the critical issue as to whether cyclin D-associated cdks are indeed required for estrogen-induced cell cycle progression has not been examined as yet. Both of these uncertainties are directly addressed by our present study, by using lovastatin to eliminate any potential mitogenic effects of the autocrine growth factors produced by the MCF-7 cells (see also reference 7) and by directly demonstrating that either neutralization of cyclin D1 or inhibition of cdk4/6 by p16 blocked the estradiol-stimulated cell cycle progression in G_1. These experiments showed that the cyclin D-cdk activity is an essential downstream component of the estradiol-dependent mitogenic cascade and the one on which the biochemically distinct pathways triggered by either polypeptide growth factors or estrogens converge to promote commitment to proceed through the late G_1 checkpoint. The key role of this cyclin in the late G_1 commitment process is also documented here by its ability to promote the S-phase onset when selectively expressed upon zinc induction in T-47D cells in the absence of upstream signals from estrogen receptors and prevented from growth factor-induced mitogenesis by lovastatin.

Overall, the data obtained in this study provide further support for the emerging candidacy of the cyclin D-cdk-p16-pRb interplay for its involvement in the restriction point machinery (4, 61, 63, 66). This G_1 checkpoint mechanism fulfills the criteria originally proposed by Pardee (51) for the R-point switch (4, 34, 61, 63, 66, 73), including its role as a growth factor sensor, its execution in mid- to late G_1, positive regulation by a labile component, and its common deregulation in oncogenesis. Apart from its molecular identity, another feature which was impossible to predict based on the original operational definition of the R-point concept (51) is the unexpected degree of complexity of this regulatory mechanism. Thus, the accumulating evidence implicates at least the multigene families of D-type cyclins and cdk inhibitors, together with cdk4, cdk6, and their pRb substrate, as the core components of this G_1 checkpoint control identified thus far (4, 9, 18, 57, 62, 63, 66). More comprehensive discussions of the role of pRb phosphorylation and its regulation by various cyclin-cdk complexes and cdk inhibitors can be found in recent review articles (4, 62, 63, 66). It is, however, likely that the list of additional regulators operating downstream (12, 22, 39, 66) and especially upstream of this central step, as suggested also by the present study, will be considerably longer. The challenge ahead is to understand the molecular basis of this core mechanism and its links with the incoming upstream cascades, which should greatly help us appreciate the global involvement of this checkpoint in switching between alternative cellular fates and as an emerging obligatory target in multistep oncogenesis.

ACKNOWLEDGMENTS

This work was supported by research grants from the Danish Cancer Society, the Czech Ministry of Health, and Novo Nordisk Foundation.

We thank Ingrid Fossar Larsen and Hanne Gamst-Andersen for supply of the dog thyroid gland tissue, Pierre Roger for kind advice on thyrocyte isolation and culture, Klaus Hansen and Michael Strauss for fruitful discussions, and Anne Lykkesfeldt, Kristian Helin, Masao Seto, Wen-Hwa Lee, Gordon Peters, Elizabeth Musgrove, and Chris Marshall for their kind gifts of various reagents.

REFERENCES

1. **Ajchenbaum, F., K. Ando, J. A. DeCaprio, and J. D. Griffin.** 1993. Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. J. Biol. Chem. **268:**4113–4119.
2. **Altucci, L. R. Addeo, L. Cicatiello, S. Dauvois, M. G. Parker, M. Truss, M. Beato, V. Sica, F. Bresciani, and A. Weisz.** 17β-estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G1-arrested human breast cancer cells. Oncogene **12:**2315–2324.
3. **Baldin, V., J. Lukas, M. Marcote, M. Pagano, and G. Draetta.** 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. **7:**812–821.
4. **Bartek, J., J. Bartkova, and J. Lukas.** The retinoblastoma protein pathway and the restriction point. Curr. Opin. Cell Biol., in press.
5. **Bartkova, J., J. Lukas, H. Müller, D. Lützhøft, M. Strauss, and J. Bartek.** 1994. Cyclin D1 protein expression and function in human breast cancer. Int. J. Cancer **57:**353–361.
6. **Bates, S., L. Bonetta, D. MacAllan, D. Parry, A. Holder, C. Dickson, and G. Peters.** 1994. CDK6 (PLSTIRE) and CDK4 (PSK-J3) are a distinct subset of

the cyclin-dependent kinases that associate with cyclin D1. Oncogene **9:**71–79.
7. **Bonapace, I. M., R. Addeo, L. Altucci, L. Cicatiello, M. Bifulco, C. Laezza, S. Salzano, V. Sica, F. Bresciani, and A. Weisz.** 1996. 17β-Estradiol overcomes a G1 block induced by HMG-CoA reductase inhibitors and fosters cell cycle progression without inducing ERK-1 and -2 MAP kinases activation. Oncogene **12:**753–763.
8. **Clarke, A. R., E. R. Maandag, M. van Roon, N. M. T. van der Lugt, M. van der Valk, M. L. Hooper, A. Berns, and H. te Riele.** 1992. Requirement for a functional RB-1 gene in murine development. Nature (London) **359:**328–330.
9. **Coats, S., W. M. Flanagan, J. Nourse, and J. M. Roberts.** 1996. Requirement of p27 Kip1 for restriction point control of the fibroblast cell cycle. Science **272:**877–880.
10. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:**841–852.
11. **DeClue, J. E., W. C. Vass, A. G. Papageorge, D. R. Lowy, and B. M. Willumsen.** 1991. Inhibition of cell growth by lovastatin is independent of ras function. Cancer Res. **51:**712–717.
12. **Degregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
13. **Dumont, J. A., F. Lamy, P. Roger, and C. Maenhaut.** 1992. Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. Physiol. Rev. **72:**667–697.
14. **Foster, J. S., and J. Wimalasena.** 1996. Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. Mol. Endocrinol. **10:**488–498.
15. **Furlamento, R. W., S. E. Harwell, and K. K. Frick.** 1994. Insulin-like growth factor-I induces cyclin D1 expression in MG63 human osteosarcoma cells in vitro. Mol. Endocrinol. **8:**510–517.
16. **Goodrich, D. W., and W.-H. Lee.** 1993. Molecular characterization of the retinoblastoma susceptibility gene. Biochim. Biophys. Acta **1155:**43–61.
17. **Hall, M., and G. Peters.** 1996. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv. Cancer Res. **68:**67–108.
18. **Harper, J. W., and S. J. Elledge.** 1996. Cdk inhibitors in development and cancer. Curr. Opin. Genet. Dev. **6:**56–64.
19. **Heldin, C.-H.** 1995. Dimerization of cell surface receptors in signal transduction. Cell **80:**213–223.
20. **Hengst, L., V. Dulic, J. M. Slingerland, M. Lees, and S. I. Reed.** 1994. A cell cycle-regulated inhibitor of cyclin-dependent kinases. Proc. Natl. Acad. Sci. USA **91:**5291–5295.
21. **Hill, C. S., and R. Treisman.** 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell **80:**199–211.
22. **Hofmann, F., and D. M. Livingston.** 1996. Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. Genes Dev. **10:**851–861.
23. **Hunter, T., and J. Pines.** 1994. Cyclins and cancer II: cyclin D and cdk inhibitors come of age. Cell **79:**573–582.
24. **Jacks, T., A. Fazeli, E. M. Schmitt, R. T. Bronson, M. A. Goodell, and R. A. Weinberg.** 1992. Effects of an Rb mutation in the mouse. Nature (London) **359:**295–300.
25. **Jakobisiak, M., S. Bruno, J. S. Skierski, and Z. Darzynkiewicz.** 1991. Cell cycle-specific effects of lovastatin. Proc. Natl. Acad. Sci. USA **88:**3628–3632.
26. **Kamb, A., N. A. Gruis, J. Weaver-Feldhaus, Q. Liu, K. Harshman, S. V. Tavtigian, E. Stockert, R. S. Day, B. E. Johnson, and M. H. Skolnick.** 1994. A cell cycle regulator potentially involved in genesis of many tumor types. Science **264:**436–440.
27. **Keyomarsi, K., L. Sandoval, V. Band, and A. B. Pardee.** 1991. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. Cancer Res. **51:**3602–3609.
28. **Kohn, J., G. H. Enders, B. D. Dynlacht, and E. Harlow.** 1995. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. Nature (London) **375:**506–510.
29. **Lamy, F., F. Wilkin, M. Baptist, J. Posada, P. R. Roger, and J. E. Dumont.** 1993. Phosphorylation of mitogen-activated protein kinases is involved in the EGF and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway. J. Biol. Chem. **268:**8398–8401.
30. **Lebowitz, P. F., J. P. Davide, and G. C. Prendergast.** 1995. Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol. Cell. Biol. **15:**6613–6622.
31. **Lee, E. Y.-H., C.-Y. Chang, N. Hu, Y.-C. J. Wang, C.-C. Lai, K. Herrup, W.-H. Lee, and A. Bradley.** 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature (London) **359:**288–294.
32. **Liu, J.-J., J.-R. Chao, M.-C. Jiang, S.-Y. Ng, J. J.-Y. Yen, and H.-F. Yang-Yen.** 1995. Ras transformation results in an elevated level of cyclin D1 and acceleration of G_1 progression in NIH 3T3 cells. Mol. Cell. Biol. **15:**3654–3663.
33. **Lukas, J., L. Aagaard, M. Strauss, and J. Bartek.** 1995. Oncogenic aberrations of $p16^{INK4/CDKN2}$ and cyclin D1 cooperate to deregulate G_1 control. Cancer Res. **55:**4818–4823.
34. **Lukas, J., J. Bartkova, M. Rohde, M. Strauss, and J. Bartek.** 1995. Cyclin D1 is dispensable for G_1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. Mol. Cell. Biol. **15:**2600–2611.
35. **Lukas, J., D. Jadayel, J. Bartkova, E. Nacheva, M. J. S. Dyer, M. Strauss, and J. Bartek.** 1994. BCL-1/cyclin D1 oncoprotein oscillates and subverts the G1 phase control in B-cell neoplasms carrying the t(11;14) translocation. Oncogene **9:**2159–2167.
36. **Lukas, J., H. Müller, J. Bartkova, D. Spitkovsky, A. A. Kjerulff, P. Jansen-Dürr, M. Strauss, and J. Bartek.** 1994. DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. J. Cell Biol. **125:**625–638.
37. **Lukas, J., M. Pagano, Z. Staskova, G. Draetta, and J. Bartek.** 1994. Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumour cell lines. Oncogene **9:**707–718.
38. **Lukas, J., D. Parry, L. Aagaard, D. J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek.** 1995. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature (London) **375:**503–506.
39. **Lukas, J., B. O. Petersen, K. Holm, J. Bartek, and K. Helin.** 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes $p16^{INK4}$-mediated growth suppression. Mol. Cell. Biol. **16:**1047–1057.
40. **Maltese, W. A.** 1990. Posttranslational modification of proteins by isoprenoids in mammalian cells. FASEB J. **4:**3319–3328.
41. **Marshall, C. J.** 1996. Ras effectors. Curr. Opin. Cell Biol. **8:**197–204.
42. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J.-Y. Kato.** 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. **14:**2066–2076.
43. **Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr.** 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell **65:**701–713.
44. **Medema, R. H., R. E. Herrera, F. Lam, and R. A. Weinberg.** 1995. Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc. Natl. Acad. Sci. USA **92:**6289–6293.
45. **Meloche, S., K. Seuwen, G. Pages, and J. Pouyssegur.** 1992. Biphasic and synergistic activation of $p44^{mapk}$ (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Mol. Endocrinol. **6:**845–854.
46. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077–2086.
47. **Migliaccio, A., M. DiDomenico, G. Castoria, A. deFalco, P. Bontempo, E. Nola, and F. Auricchio.** 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. **15:**1292–1300.
48. **Musgrove, E. A., J. A. Hamilton, C. S. L. Lee, K. J. E. Sweeney, C. K. W. Watts, and R. L. Sutherland.** 1993. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. Mol. Cell. Biol. **13:**3577–3587.
49. **Musgrove, E. A., C. S. L. Lee, M. F. Buckley, and R. L. Sutherland.** 1994. Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. Proc. Natl. Acad. Sci. USA **91:**8022–8026.
50. **Nevins, J. R.** 1992. E2F, a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
51. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246:**603–608.
52. **Parry, D., S. Bates, D. J. Mann, and G. Peters.** 1995. Lack of cyclin D-cdk complexes in RB-negative cells correlates with high levels of $p16^{Ink4/MTS1}$ tumour suppressor gene product. EMBO J. **14:**503–511.
53. **Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts, and M. Pagano.** 1995. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15:**2612–2624.
54. **Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, J.-Y. Kato, D. Bar-Sagi, M. F. Roussel, and C. J. Sherr.** 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. **7:**1559–1571.
55. **Resnitzky, D., M. Gossen, H. Bujard, and S. I. Reed.** 1994. Acceleration of the G_1/S phase transition by expression of cyclins D1 and E with inducible system. Mol. Cell. Biol. **14:**1669–1679.
56. **Resnitzky, D., and S. I. Reed.** 1995. Different roles for cyclins D1 and E in regulation of the G_1-to-S transition. Mol. Cell. Biol. **15:**3463–3469.
57. **Rivard, N., G. L'Allemain, J. Bartek, and J. Pouyssegur.** 1996. Abrogation of p27Kip1 by cDNA antisense suppresses quiescence (G0 state) in fibroblasts. J. Biol. Chem. **271:**18337–18341.
58. **Serrano, M., E. Gomez-Lahoz, R. A. DePinho, D. Beach, and D. Bar-Sagi.** 1995. Inhibition of Ras-induced proliferation and cellular transformation by $p16^{INK4}$. Science **267:**249–252.
59. **Serrano, M., G. J. Hannon, and D. Beach.** 1993. A regulatory motif in cell-cycle control causing specific inhibition of cyclin D/cdk4. Nature (London) **366:**704–707.
60. **Sherr, C. J.** 1993. Mammalian G1 cyclins. Cell **73:**1059–1065.
61. **Sherr, C. J.** 1994. G1 phase progression: cycling on cue. Cell **79:**551–555.
62. **Sherr, C. J., and J. M. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-

dependent kinases. Genes Dev. **9:**1149–1163.
63. **Strauss, M., J. Lukas, and J. Bartek.** 1995. Unrestricted cell cycling and cancer. Nat. Med. **1:**1245–1246.
64. **Tam, S. W., A. M. Theodoras, J. W. Shay, G. F. Draetta, and M. Pagano.** 1994. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with cdk4 is required for cyclin D1 function in G1 progression. Oncogene **9:**2663–2674.
65. **Watts, C. K. W., A. Brady, B. Sarcevic, A. deFazio, E. A. Musgrove, and R. L. Sutherland.** 1995. Antiestrogen inhibition of cell cycle progression in breast cancer cells is associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation. Mol. Endocrinol. **9:**1804–1813.
66. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
67. **Wiman, K. G.** 1993. The retinoblastoma gene: role in cell cycle control and cell differentiation. FASEB J. **7:**841–845.
68. **Winston, J. T., S. R. Coats, Y.-Z. Wang, and W.J. Pledger.** 1996. Regulation of the cell cycle machinery by oncogenic ras. Oncogene **12:**127–134.
69. **Winston, J. T., and W. J. Pledger.** 1993. Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. Mol. Biol. Cell. **4:**1133–1144.
70. **Withers, D. J., S. R. Bloom, and E. Rozengurt.** 1995. Dissociation of cAMP-stimulated mitogenesis from activation of the mitogen-activated protein kinase cascade in Swiss 3T3 cells. J. Biol. Chem. **270:**21411–21419.
71. **Won, K. A., Y. Xiong, D. Beach, and M. Z. Gilman.** 1992. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA **89:**9910–9914.
72. **Wyke, A. W., M. C. Frame, D. A. F. Gillespie, A. Chudleigh, and J. A. Wyke.** 1995. Mitogenesis by v-src: fluctuations throughout G1 of classical immediate early AP-1 and mitogen-activated protein-kinase responses that parallel the need for the oncoprotein. Cell Growth Differ. **6:**1225–1234.
73. **Zetterberg, A., O. Larsson, and K. G. Wiman.** 1995. What is the restriction point? Curr. Opin. Cell Biol. **7:**835–842.