Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G₁ exit

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The cyclin-dependent kinases cdk2 and cdk3 are required for the G₁-S transition in mammalian cells. Here we show that G₁ arrest induced by the corresponding dominant-negative mutants of these enzymes, cdk2dn or cdk3dn, is resistant to the action of SV40 T antigen (T). In the presence of cdk2dn, T released active E2F from negative control by pRb and its related family members (pocket proteins) but failed to induce S-phase. Therefore, among other targets, cdk2 also phosphorylates nonpocket protein substrates in promoting S-phase entry, and T does not mimic all cdk2 functions. In the presence of cdk3dn, however, T failed to induce cell cycle progression or stimulate E2F-dependent transcription activity. Dominant-negative cdk3 inhibited E2F-1, E2F-2, and, less significantly, E2F-3, but not E2F-4 transcription activity. The inhibition occurred in a pRb-independent manner and did not affect the DNA-binding capacity of the transcription factor. Cdk3 bound specifically to E2F-1/DP-1 complexes *in vivo*, most likely through DP-1. Thus, cdk3 function contributes to the activation of E2F-1, E2F-2, and partially, E2F-3 and, thereby, participates in the process of S-phase entry.

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The proliferation of eukaryotic cells is controlled by a complex system of growth-stimulating and retarding signals. The best characterized control points in the cell cycle are the G₁-S and the G₂-M transitions, which commit a cell to DNA replication and mitosis, respectively. In yeast (Forsburg and Nurse 1991) it is clear that both transitions are regulated by a single protein kinase, Cdc2 in *Schizosaccharomyces pombe* and Cdc28 in *Saccharomyces cerevisiae*. These enzymes are activated, in part, by associating with the appropriate cyclin regulatory subunits at different times in the cell cycle. In contrast, in higher eukaryotes, multiple cyclin-dependent kinases (cdks) exist, and their activation, too, is regulated in part by binding to different cyclin regulatory subunits.

During mammalian G₁, D-type cyclins associate with cdk4 and cdk6, and cyclin E interacts with cdk2 (Draetta 1994). At G₁-S, cyclin A is also synthesized, and its primary partner is cdk2 (Pines 1993).

Cyclin D- and cyclin E-associated kinases participate in G₁ progression and in the transition from G₁ into S-phase. Both are active in the phosphorylation of the tumor suppressor protein pRb (Weinberg 1995), a crucial G₁ process that leads to the release and activation of one or more members of the E2F transcription factor family. E2F release from pRb is an essential event in the activa-

tion of genes required for S-phase entry (Nevins 1992; Lam and La Thangue 1994).

Another member of the cdk family, cdk3, was isolated as a cdc2 homolog. Like cdc2 and cdk2, the cdk3 gene was shown to complement *cdc28* mutants of *S. cerevisiae* (Meyerson et al. 1992). The cyclin partner of cdk3 is not yet known, but biological studies, using a dominant-negative mutant of cdk3, imply that its function, like that of cdk2, is required for the mammalian G₁-S transition. Indeed, overproduction of dominant-negative mutant forms of cdk2 or cdk3 led to G₁ arrest (van den Heuvel and Harlow 1993). Despite their structural similarity, the block could be overcome specifically by the corresponding, but not the other wild-type enzyme, indicating that these two kinases operate on different pathways.

In an effort to understand the pathways controlled by cdk2 and cdk3 at the G₁-S transition, we asked whether each contributes to the pRb phosphorylation process and, thereby, promotes E2F release.

Results

Dominant-negative mutants of cdk2 and cdk3 induce SV40 T antigen-resistant G₁ arrest

SV40 T antigen (T) is known to bind and functionally inactivate unphosphorylated pRb, as well as the related pocket proteins p107 and p130 (DeCaprio et al. 1988; Dyson et al. 1989; Ewen et al. 1989; Ludlow et al. 1989;

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Hannon et al. 1993; Wolf et al. 1995; Zalvide and De-Caprio 1995). T binding to pRb promotes release of the transcription factor E2F. If cdk2 or cdk3, or both, were primarily involved in the functional inactivation of pRb and if E2F release were the downstream event necessary for S-phase entry, then G₁ arrest, induced by overproduction of either cdk2dn or cdk3dn, might well be overcome by T, which in turn, might be expected to release active E2F from pocket proteins. The latter is a known S-phase promoting event (Nevins 1992; Lam and La Thangue 1994; Weinberg 1995).

As shown in Figure 1A, the state of G₁ arrest induced in U2OS cells by both cdk2 and cdk3 dominant-negative mutants was not overcome by increasing amounts of T. T was found to be functionally active in this experimental setting, because, as expected, it overrode a p16-induced G₁ block (data not shown). In keeping with this result, the ectopic overproduction of both dominant-negative mutants induced G₁ arrest in VA13, a line of SV40-transformed human fibroblasts that produces abundant T (Fig. 1C). Given these observations, it is possible that cdk2 and cdk3 license G₁ exit by a mechanism that includes steps downstream of or, conceivably, unrelated to the pocket protein-E2F pathway. Alternatively, in the presence of high levels of either mutant cdk species, T may not release E2F in an active form from the relevant pocket proteins.

T releases active E2F in the presence of cdk2dn but not cdk3dn

In an attempt to understand the resistance of the cdk2dn and cdk3dn G₁ blocks to T, we investigated the effect of the mutant kinases on the *trans*-activation potential of endogenous E2F in the presence and absence of T. The question was whether T can promote the release of active E2F from the relevant pocket proteins under these conditions.

Specifically, the activity of an E2F luciferase reporter plasmid was analyzed in U2OS cells. When cdk2dn or cdk3dn was cotransfected into these cells, there was significant inhibition of endogenous E2F activity. A similar effect accompanied cotransfection of the pRb mutant pRb-Δp34 (Hamel et al. 1990), which binds E2F efficiently but cannot be phosphorylated by cdks (Fig. 2A). Therefore, it is resistant to the normal cell cycle-dependent phosphorylation of pRb and does not release E2F when exposed to the G₁ cyclin/cdk complexes responsible for timely pRb phosphorylation at G₁-S. The overproduction of cdk2dn, cdk3dn, or pRb-Δp34 had no significant effect on basal reporter activity, as measured from a reporter bearing mutated E2F sites (data not shown). Hence, the inhibitory activity of these mutant proteins on wild-type reporter activity is E2F dependent.

To monitor the ability of T to induce E2F-dependent

Figure 1. Overproduction of dominant negative cdk2 or cdk3 induces G₁ arrest resistant to T. (A) U2OS cells were transfected with a CD19 expression vector (1 μg) together with pCMV (20 μg), pCMV-cdk2dn-HA (20 μg), or pCMV-cdk3dn-HA (20 μg). Increasing amounts of pSG5-T (2.5 μg, lanes 2,6,10; 5 μg, lanes 3,7,11; 10 μg, lanes 4,8,12) were cotransfected. The total amount of pSG5 DNA was kept constant in all samples (10 μg/sample) by adding, where needed, pSG5 plasmid DNA. Cells were harvested 48 hr post-transfection, stained for CD19 and DNA content, and analyzed by flow cytometry. The percentage of CD19-positive cells in G₁ was plotted for each sample. (B) Aliquots of lysate from cells analyzed in A were immunoblotted with an anti-T monoclonal antibody (PAB419; *top*) and with an anti-HA monoclonal antibody (12CA5; *bottom*) to determine the expression of the relevant transfected genes. (C) VA13 cells (a line of WI38 cells transformed by SV40) were transfected with a CD19-expressing vector (1 μg) together with either pCMV (20 μg, lane 1), increasing amounts of pCMV-cdk2dn-HA (10 μg, lane 2; 20 μg, lane 3), or increasing amounts of pCMV-cdk3dn-HA (10 μg, lane 4; 20 μg, lane 5). The amount of pCMV DNA was kept constant in all samples (20 μg/sample). Cells were harvested 48 hr post-transfection, stained for CD19 and DNA content, and analyzed by flow cytometry. The percentage of CD19-positive cells in G₁ was plotted. (D) Aliquots of lysate from VA13 cells analyzed in C were immunoblotted with an anti-HA monoclonal antibody (12CA5) to determine the expression of HA-tagged cdks.

Figure 2. T activates E2F in the presence of cdk2dn but not in the presence of cdk3dn. (A) U2OS cells were transfected with a luciferase reporter plasmid containing either three wild-type or three mutated E2F sites, 3xwt-E2F-Luc and 3xmut-E2F-Luc, respectively (2 μ g). pCMV (20 μ g), pCMV-cdk2dn-HA (20 μ g), pCMV-cdk3dn-HA (20 μ g), or pCMV-Puro-HA-pRb- Δ p34 (5 μ g) were cotransfected, where indicated. The level of pCMV DNA was constant in all samples (20 μ g/sample). pCMV- β -gal (2 μ g) was also cotransfected, in each case to permit normalization of the luciferase values to the β -galactosidase activity and thereby, correct for variations in transfection efficiency. The relative amount of luciferase activity detected after transfection was plotted. (B) U2OS cells were transfected with a luciferase reporter plasmid, 3xwt-E2F-Luc

or 3xmut-E2F-Luc, together with either pCMV (20 μ g), pCMV-cdk2dn-HA (20 μ g), or pCMV-cdk3dn-HA (20 μ g). For each of these combinations, three different conditions were tested. Specifically, pSG5 (6 μ g), pSG5-K1 (6 μ g), or pSG5-T (6 μ g) was added to the transfection mixture. The ratio of the luciferase activity measured in the presence of K1 or T and the activity measured in their absence was calculated in each case to determine the induction of E2F-dependent transcription activity by K1 or T. The average values of at least three independent experiments were plotted. The vertical bars indicate standard deviation.

transcription, the ratio between luciferase activity measured in the presence of T and that measured in its absence was determined. T overcame the inhibition of E2F-dependent transcription activity only in cdk2dn cotransfected cells (Fig. 2B). The E2F transcription activity detected in this particular setting was as great as or, in certain cases, even greater than that measured in cycling cells (data not shown), implying that the activity induced by T was most likely not the limiting factor for cell cycle progression in this case. In contrast, no T-dependent E2F activation was observed in cdk3dn cotransfected cells. A mutant of T, which cannot bind pocket proteins, K1 (DeCaprio et al. 1988), failed in any setting to activate E2F, as expected. Similar quantities of T and K1 were present in these transfected cell populations, as determined by immunoblotting (data not shown), indicating that the E2F activation effect of T in cdk2dn cotransfected cells can be linked to its ability to displace active E2F from pocket proteins. Thus, with respect to its effect on E2F activity, T led to different outcomes in these two settings.

In mock (pCMV)-transfected U2OS cells, T also failed to increase E2F transcription activity. In asynchronously growing U2OS cells, much of the E2F pool is uninhibited by pocket proteins. Experimental evidence supporting this notion is provided in Figure 4 (lanes 1–4, below), where an extract of mock-transfected U2OS cells lacked significant quantities of G₁-specific, pocket protein-E2F DNA-binding complexes (Shirodkar et al. 1992). Hence, T would not be expected to stimulate E2F activity in this setting.

These results strongly suggest that in part, cdk2 and cdk3, regulate different steps in the mammalian G₁-S transition, in keeping with previous observations (van

den Heuvel and Harlow 1993). In particular, their contributions to the control of the pRb-E2F pathway appear to be different.

cdk3dn inhibits E2F-1 in a pRb-independent manner

In an attempt to understand better the mechanism by which cdk3dn inhibits E2F-dependent transcriptional activity, we compared the *trans*-activation potential of wild-type and certain mutant E2F-1 species in the presence of cdk3wt and of cdk3dn. The effect of either wild-type or dominant-negative cdk3 on the *trans*-activation potential of E2F-1 was first tested by cotransfecting E2F-1 and DP-1 expression plasmids into U2OS cells together with a suitable reporter plasmid. Although E2F-1 transcriptional activity was largely unaffected by the presence of wild-type cdk3, dominant-negative cdk3 was clearly inhibitory (Fig. 3A).

We also tested the effect of cdk3dn on the *trans*-activation potential of an E2F-1 deletion/substitution mutant, the *trans*-activating domain of which was substituted by that of VP16, E2F-1-VP16. This chimeric transcription factor, which cannot bind pRb, was largely inactive in the presence of cdk3dn but not in the presence of the wild-type enzyme (Fig. 3A). Therefore, the cdk3dn-dependent inhibition of E2F-1 activity does not depend on the formation of pRb/E2F-1 complexes nor does it require the existence of the native E2F-1 *trans*-activating domain.

Because E2F-1 was shown to bind *in vivo* to cyclin A/cdk2 through a sequence in its amino-terminal region (Krek et al. 1994), we asked whether cdk3, together with its unknown cyclin partner, modulates the E2F-1 transcriptional activity by direct interaction with E2F-1

Figure 3. Dominant-negative cdk3 inhibits E2F-1 in a pRb-independent manner. (A) U2OS cells were transfected with a luciferase reporter plasmid, 3xwt-E2F-Luc or 3xmut-E2F-Luc, respectively (2 μ g). pCMV- β -gal (2 μ g) and pRcCMV-HA-E2F-1 (4 μ g), pRcCMV-HA-E2F-1-VP16 (4 μ g), pCMV-HA-E2F-1- Δ 24 (4 μ g), or pRcCMV-HA-E2F-1-(1-368) (4 μ g) were cotransfected with pRcCMV-T7-DP-1 (4 μ g), and with pCMV-cdk3wt-HA (20 μ g) or pCMV-cdk3dn-HA (20 μ g), where indicated. pCMV was added, where necessary, to transfect an equivalent amount of CMV promoter in every setting. The luciferase values were corrected to β -galactosidase to control for transfection efficiency. The activity measured with the mutant reporter was set at 1 in each experiment and used to normalize the activities measured in the presence of the wild-type reporter. The average values of at least three independent experiments were plotted. Vertical bars indicate the standard deviation in the amplitude of the E2F activities in each setting among different experiments. In each experiment, the activity measured in the presence of cdk3dn was reproducibly and significantly lower than that measured with cdk3wt, except in the case of E2F-1-(1-368). (B) To compare the *trans*-activation capacity of different members of the E2F family in the presence of either wild-type or dominant-negative cdk3, U2OS cells were transfected, as above, using pRcCMV-HA-E2F-1 (4 μ g), pRcCMV-HA-E2F-2 (4 μ g), pRcCMV-HA-E2F-3 (4 μ g), or pRcCMV-HA-E2F-4 (4 μ g), together with pRcCMV-T7-DP-1 (4 μ g), where indicated. The reporter activity of each E2F family member measured in the presence of cdk3wt was set to 1 in each experiment and used to normalize the activity measured in the presence of cdk3dn. The average values of at least three independent experiments were plotted; the vertical bars indicate standard deviation.

through the same sequence. cdk3dn was found to inhibit the cyclin A-binding mutant of E2F-1, E2F-1- Δ 24, as well as wt-E2F-1 (Fig. 3A), indicating that cdk3 does not use the cyclin A-binding domain of E2F-1 to regulate the transcription activity of the latter.

To rule out nonspecific inhibition of transcription by cdk3dn, the effect of cdk3wt and cdk3dn on the basal level of the reporter system, measured in the presence of an E2F-1 *trans*-activation mutant, E2F-1-(1-368), was analyzed. It was unaffected (Fig. 3A).

We then asked whether cdk3dn inhibits the *trans*-activation potential of other members of the E2F family of transcription factors. A comparison of the transcriptional activity measured in the presence of cdk3wt or cdk3dn indicated that the *trans*-activation potential of E2F-1, E2F-2 and, to a lesser extent, E2F-3, was inhibited significantly by cdk3dn (Fig. 3B). On the other hand, E2F-4, which is different from E2F-1, E2F-2, and E2F-3 in that it normally interacts with p107 and p130 and, less so, with pRb (Beijersbergen et al. 1994; Ginsberg et al. 1994; Vairo et al. 1995), was found to *trans*-activate an E2F-dependent promoter equally well in the presence of either cdk3wt or cdk3dn (Fig. 3B). This observation suggests that the transcription activity of members of the

E2F family, which interact primarily with pRb, is controlled not only by pRb, but also by cdk3.

E2F-1 retains its DNA-binding capacity in the presence of cdk3dn

The capacity of E2F-1/DP-1 heterodimers to bind an E2F site-containing oligonucleotide in the presence of cdk3dn was investigated in an electrophoretic mobility-shift assay (EMSA) (Fig. 4). Hemagglutinin (HA)-tagged E2F-1 was transfected into U2OS cells alone in the presence of wild-type cdk3 or together with the dominant-negative version of this enzyme. The ability of HA-tagged E2F-1 to bind an oligonucleotide corresponding to a segment of the dihydrofolate reductase (DHFR) promoter (Wade et al. 1992) was probed by EMSA using an anti-HA antibody to identify complexes containing HA-E2F-1 (Krek et al. 1993). As shown in Figure 4, the DNA binding capacity of comparably tagged E2F-1-containing complexes was perturbed by anti-HA antibody in all of the different transfection combinations. HA-E2F-1 was detected both as a fast migrating form A ("free E2F") and as a slower migrating pRb/E2F-DP complex, known as complex B (Shirodkar et al. 1992). In keeping with this

Figure 4. E2F-1 DNA-binding capacity is not affected by cdk3dn. U2OS cells were transfected with either pCMV (20 μ g), pCMV-cdk3wt-HA (20 μ g), or pCMV-cdk3dn-HA (20 μ g), and, where indicated, with pRcCMV-HA-E2F-1 (4 μ g). The E2F-DNA-binding complexes were characterized either by DNA-binding competition, adding 100 ng of cold wild-type oligonucleotide (lane 2), or 100 ng of cold oligonucleotide containing a mutated E2F site (lane 3), or by the addition of specific previously characterized antibodies (Shirodkar et al. 1992; Krek et al. 1993): 0.5 μ g of affinity-purified anti-HA antibody (12CA5) (lane 4,6,8,10) or 2 μ l of anti-pRb monoclonal antibody (C36) (lane 12,14,16). The DNA-bind-

ing complexes marked A, B, and C correspond to previously characterized species (Shirodkar et al. 1992). They are, respectively, free E2F, a pRb-containing complex, and an S-phase specific p107-containing complex.

designation the latter was supershifted by the pRb-specific monoclonal antibody C36. In the presence of cdk3dn both complexes were readily apparent, suggesting strongly that E2F-1/DP-1 heterodimers had formed (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993). This was confirmed by a supershift effect obtained with anti-DP-1 antibodies (data not shown). Therefore, the inhibition of E2F-1 *trans*-activating activity by dominant-negative cdk3 is not likely a result of defective heterodimerization or DNA binding.

cdk3 associates in vivo with an E2F-1-containing complex through DP-1

To understand better the mode of action of cdk3 in the control of E2F-dependent transcription activity, we asked whether cdk3 contributes to the regulation of E2F-1 *trans*-activation function by direct interaction with the transcription factor. A monoclonal antibody raised against E2F-1, SQ41, coimmunoprecipitated E2F-1, DP-1, and a band that comigrated with HA-cdk3 from extracts of cells transfected with E2F-1, DP-1, and either HA-cdk3wt or HA-cdk3dn, (Fig. 5A, lanes 3,4). The coimmunoprecipitated band comigrated with HA-cdk3, both when the latter was immunoprecipitated with an anti-HA antibody 12CA5, or with a polyclonal antibody raised against the carboxyl terminus of cdk3, Y-20 (Fig. 5A, lanes 7,10). The anti-cdk3 antibody also immunoprecipitated a band comigrating with the 33-kD marker. We believe this to be a form of cdk3 truncated at the carboxyl terminus, as it was not recognized by the anti-HA antibody, the target epitope for which it is at the carboxyl terminus. It was, however, present in anti-cdk3 immunoprecipitates obtained from denatured extracts, consistent with the speculation that it is a shortened form of cdk3.

The ~35-kD band that coimmunoprecipitated with E2F-1 and comigrated with HA-cdk3 was an associated protein. Immunoprecipitation performed with a monoclonal antibody to E2F-1 SQ41 yielded the band of inter-

est only when native, and not denatured, extracts were used (Fig. 5B, lanes 3,4). cdk3 was present in similar amounts in native as well as in denatured extracts, as shown by immunoprecipitation experiments performed with the anti-cdk3 antibody Y-20 (Fig. 5B, lanes 8,9). In addition, the band comigrating with HA-cdk3 was detected after anti-E2F-1 immunoprecipitation, only when native extracts from cells transfected with HA-cdk3 were used (Fig. 5B, cf. lanes 2,3,5). This suggests that the band corresponds to HA-cdk3. Moreover, its intensity was much greater when an E2F-1 cDNA was expressed ectopically, indicating that its appearance is most likely not the product of nonspecific binding of the SQ-41 antibody to HA-cdk3 (Fig. 5B, cf. lanes 3,5,8,10).

E2F-1 interacts stably with cyclin A/cdk2 (Krek et al. 1994). To test the possibility that cdk3 associates with E2F-1 through the cyclin A-binding domain of the latter, we carried out an E2F-1 and a cdk3 immunoprecipitation on extracts of cells transfected by an E2F-1 cyclin A-binding mutant, E2F-1- Δ 24. Consistent with the observation that cdk3dn inhibits the *trans*-activation function of E2F-1- Δ 24 (see Fig. 3A), a protein comigrating with HA-cdk3 was clearly coimmunoprecipitated together with E2F-1- Δ 24 and DP-1 by the anti-E2F-1 antibody SQ41 (Fig. 5C, cf. lanes 3 and 4 with 7 and 8).

To learn whether the ~35-kD coprecipitating protein was indeed cdk3, a reimmunoprecipitation experiment was performed with an anti-cdk3 antibody, Y-20. This antibody, but not an unrelated, polyclonal control antibody, reimmunoprecipitated HA-cdk3 both from immunocomplexes obtained with Y-20 itself, or with the anti-E2F-1 antibody SQ41 when HA-cdk3 was cotransfected with E2F-1- Δ 24 and DP-1 (Fig. 5D, lanes 1-10). In agreement with what was observed previously (Fig. 5B, lanes 3,5,8,10), consistently less HA-cdk3 was present in an anti-E2F-1 immunocomplex generated in cells in which E2F-1 was produced endogenously and not ectopically (Fig. 5D, lanes 11-14, specifically compare the ~35-kD band representing HA-cdk3 in lanes 8 and 14).

Immunoprecipitation of T7-DP-1 using a monoclonal

antibody raised against the T7 tag also led to the coimmunoprecipitation of HA-cdk3 when both were synthesized ectopically (Fig. 6, cf. lanes 1–6 with lanes 10–15). Moreover, overproduction of E2F-1 was not necessary for

the coimmunoprecipitation of T7-DP-1 and HA-cdk3 (Fig. 6, lane 5), suggesting that cdk3 interacts directly with DP-1. When increasing amounts of T7-DP-1 were cotransfected with a constant amount of HA-cdk3, anti-

Figure 5. (See facing page for legend.)

Figure 6. cdk3 interacts with E2F-1 through its heterodimeric partner DP-1. U2OS cells were transfected with pRcCMV-HA-E2F-1-Δ24 (10 μg), pRcCMV-T7-DP-1 (10 μg, lanes 2,3,5,11,12,14; 2 μg, lanes 7,16; 4 μg, lanes 8,17; 8 μg, lanes 9,18), and pCMV-cdk3dn-HA (10 μg), as indicated. Equivalent amounts of extract obtained from the same metabolically labeled cells were subjected to immunoprecipitation with a monoclonal antibody raised against the T7 tag (lanes 1–9) and with a polyclonal antibody raised against cdk3, Y-20 (lanes 10–18).

T7 immunoprecipitation yielded increasing amounts of both T7-DP-1 and of HA-cdk3 (Fig. 6, cf. lanes 7–9 with lanes 16–18), indicating that DP-1, and not endogenous E2F-1, is the limiting factor for the association of cdk3 and the heterodimeric transcription factor. This association proved to be relatively cdk3 specific, as dominant-negative cdk2 did not coimmunoprecipitate in experiments analogous to those in which E2F-1-Δ24/DP1-cdk3 complexes were detected (data not shown).

The polyclonal antibody raised against cdk3, Y-20, did immunoprecipitate HA-tagged cdk3, as well as one or two bands with a SDS-PAGE mobility comparable to that of T7-DP-1, when extracts from cells transfected

with HA-cdk3 and T7-DP-1 were used but not when the latter was omitted or when the extract was denatured before immunoprecipitation (Fig. 5B, cf. lanes 8–10; Fig. 5C, cf. lanes 7 and 8). This observation represents additional evidence consistent with a proposed *in vivo* interaction between cdk3 and DP-1.

Discussion

The functions of cdk2 and cdk3, both known to be necessary for S-phase entry (van den Heuvel and Harlow 1993), were analyzed vis à vis the regulation of the pRb-E2F pathway. Dominant-negative mutants of these en-

Figure 5. Cdk3 associates with an E2F-containing complex *in vivo*. (A) U2OS cells transfected transiently with pCMV or with expression plasmids for the indicated proteins—pSG5-E2F-1 (10 μg), pRcCMV-HA-E2F-1 (10 μg), pRcCMV-T7-DP-1 (10 μg), pCMV-cdk3wt-HA (10 μg), pCMV-cdk3dn-HA (10 μg)—were metabolically labeled with [³⁵S]methionine. Cell extracts were immunoprecipitated with a monoclonal antibody raised against E2F-1, SQ41 (lanes 1–4); an anti-HA monoclonal antibody, 12CA5 (lanes 5–7); or a polyclonal antibody raised against the carboxyl terminus of cdk3, Y-20 (lanes 8–10). Immune complexes were resolved by SDS-PAGE. (B) U2OS cells transfected with pRcCMV-HA-E2F-1 (10 μg), pRcCMV-T7-DP-1 (10 μg), and/or pCMV-cdk3dn-HA (10 μg), as indicated, were labeled metabolically with [³⁵S]methionine and analyzed as above. Equivalent amounts of cell extract obtained from the same metabolically labeled cells were used for immunoprecipitations with a monoclonal antibody raised against E2F-1, SQ41 (lanes 1–5), or with a polyclonal antibody against cdk3, Y-20 (lanes 6–10). The extract obtained from cells transfected with all three plasmids (lanes 3,4,8,9) was subjected to immunoprecipitation either in its native (lanes 3,8) or in its denatured state (lanes 4,9). (C) Equivalent amounts of cell extract obtained from the same metabolically labeled cells, transfected with pRcCMV-HA-E2F-1-Δ24 (10 μg), pRcCMV-T7-DP-1 (10 μg), or pCMV-cdk3dn-HA (10 μg), as indicated, were analyzed as above, after immunoprecipitation with a monoclonal antibody raised against E2F-1, SQ41 (lanes 1–4) and with a polyclonal antibody against cdk3, Y-20 (lanes 5–8). (D) Equivalent amounts of cell extracts obtained from metabolically labeled cells, transfected with pRcCMV-HA-E2F-1-Δ24 (10 μg), pRcCMV-T7-DP-1 (10 μg), and/or pCMV-cdk3dn-HA (10 μg), as indicated, were used for an immunoprecipitation–reimmunoprecipitation experiment. One fifth of the immunocomplex obtained with anti-E2F-1 (SQ41) or anti-cdk3 (Y-20) antibody in the first immunoprecipitation (1st) was analyzed as above; the remaining material was subjected to reimmunoprecipitation with the indicated antibodies (2nd) before analysis by SDS-PAGE. In the reimmunoprecipitation experiment, a rabbit anti-mouse polyclonal antibody (lane 10) was used as a negative control for the rabbit anti-cdk3 polyclonal antibody Y-20. Lanes 11 and 12 represent a longer exposure of lanes 1 and 2, for comparison with an equivalently long exposure of lanes 13 and 14, which shows the results of a similar immunoprecipitation obtained with an extract from cells overproducing cdk3dn. The data imply that ectopically produced cdk3 associates with endogenous E2F-1.

zymes, cdk2dn and cdk3dn (van den Heuvel and Harlow 1993), were used as functional probes, along with T. T can inactivate pRb and related proteins functionally, promotes the release of active E2F from the latter and is a known stimulant of G₁ exit. The ability of T to override a G₁ block induced by each of the dominant-negative mutant enzymes was tested, in part to learn whether one, the other, or both kinase activities function upstream of E2F.

Both dominant-negative mutants induced a G₁ block resistant to the action of T. However, T did release active E2F from pocket protein negative control in the presence of cdk2dn, but not cdk3dn. In keeping with earlier biological results, this is the first evidence that cdk2 and cdk3 operate, at least in part, on different biochemical targets (van den Heuvel and Harlow 1993).

The fact that cdk2dn led to G₁ arrest in the presence of T, even when E2F was activated, suggests that cdk2 regulates multiple events required for the G₁-S transition of the cell cycle. A subset of these actions is unrelated to or downstream from the events of pRb phosphorylation and concomitant E2F release. Most likely cdk2, in the presence of cyclin E (Koff et al. 1991, 1992; Dulic et al. 1992), phosphorylates at least one critical target, other than pRb or E2F, the function of which is insensitive to the action of T (Fig. 7).

This hypothesis is compatible with and is supported by other recent observations. For example, microinjection of anti-cyclin E antibodies, but not anti-cyclin D1 antibodies, induced G₁ arrest even in cells lacking pRb function (Ohtsubo et al. 1995). Moreover, overproduction of cyclin E or cyclin D1 led to G₁ shortening in both cases, and the combination of both cyclins resulted in an additive effect. However, only cyclin D1 accelerated pRb phosphorylation, suggesting that cyclin E controls a different rate-limiting event necessary for the G₁-S transition (Resnitzky and Reed 1995).

On the other hand, in contrast with our observations and those reported above, others have reported that overproduction of E2F-1 led to DNA synthesis in the presence of dominant-negative cdk2 in an established rat embryo fibroblast cell line (DeGregori et al. 1995). E2F-1 was also shown to induce cell cycle progression of γ -irradiated cells in the absence of cdk2 kinase activity (DeGregori et al. 1995), implying that E2F transcription activity is sufficient for S-phase induction and operates downstream of cdk2 kinase activity. This discrepancy with the data reported here and with the results of others (Ohtsubo et al. 1995; Resnitzky and Reed 1995) may in part be a product of the use of different experimental conditions. Moreover, given that stable E2F-1 binding to functional cyclin A/cdk2 is essential for avoiding S-phase arrest and death (Krek et al. 1995), one wonders whether the DNA replicative activity seen by DeGregori et al. (1995) constitutes a normal proliferative event. In view of the possibility that it does not, it may be inappropriate to draw any firm conclusion about the ability of E2F-1 to induce DNA synthesis in the absence of cdk2 kinase activity.

Overproduction of cdk3dn also led to a T-resistant G₁

Figure 7. Alternative models summarizing the relevant observations. Both models indicate the distinct roles of cdk2 and cdk3 at the G₁-S transition according to the results reported in this paper. It is proposed that cdk2 is active in the phosphorylation of pRb and, therefore, in the release of E2F. However, it may also control additional events, the nature of which are still unknown, necessary for entry into S-phase and insensitive to the action of SV40 T antigen. It is proposed that cdk3 contributes by associating with E2F through DP-1 to the activation of E2F, thereby licensing events necessary for S-phase entry. Additional actions of cdk3 are also possible. Whether cdk3 acts before or after the release of E2F from pRb could not be determined from the results presented here. Hence, two possible model pathways are presented. In one, cdk3 acts after E2F is released from pRb (*top*). In the other, cdk3 acts before to its release (*bottom*), affecting either the E2F-DP heterodimer or the E2F heterodimer present in the E2F-DP-pRb complex. The designations active and inactive refer to the ability of an E2F-DP heterodimer to activate transcription once bound to a cognate E2F DNA-binding site.

block, but in this case, there was no T-dependent activation of E2F transcription activity. Moreover, cdk3dn inhibition of E2F-dependent transcription activity occurred even when the target E2F-1 species was defective in binding pocket proteins and cyclin A or when it contained a foreign *trans*-activating domain. Inhibition was not a result of failure of E2F-1 to bind to DNA or to heterodimerize with its DP partner. A general transcription suppression effect of cdk3dn can also be ruled out, because cdk3dn had no effect on the basal level of the E2F-dependent reporter system measured in the presence of an E2F-1 *trans*-activation mutant.

In this regard, a role for cdk3 in the regulation of E2F

transcription activity was restricted to the members of the family that are primarily targeted by pRb, E2F-1, E2F-2 and, to a minor extent, E2F-3. The *trans*-activation function of a member of the family that interacts with p107 and p130, E2F-4, was the same in the presence of wild-type or dominant-negative cdk3. Hence, cdk3 has a selective functional effect on certain E2F family members, activating primarily those species that operate under pRb control. Thus, the activity of these family members is not only controlled by pRb, but also by cdk3 (Fig. 7). Given the dual control and the known role of both pRb and cdk3 in regulating G₁ exit, one could speculate that these three E2F species normally operate, at least in part, in promoting G₁ exit.

cdk3 in its wild-type or dominant-negative form interacts specifically with an E2F-1-containing complex *in vivo*. At a minimum, given the results shown in Figure 6, this interaction appears to depend on cdk3/DP-1 binding. Thus far it has not been possible to detect cdk3-dependent phosphorylation of DP-1 *in vitro*. The cyclin A-dependent kinase associated with endogenous E2F-1 bound in turn to DP-1 constitutes a background activity against which it was impossible to detect cdk3 activity *in vitro* (F. Hofmann and D.M. Livingston, unpubl.). Indeed, it should be noted that the detailed biochemical linkage between cdk3 action and E2F activation is not yet known.

Taken together these results suggest that E2F-1, E2F-2 and, to a lesser extent, E2F-3 transcription activity is regulated not only by pRb but also by cdk3, which interacts with the transcription factor via DP-1. E2F-4 transcription activity was not impaired in the presence of non-functional cdk3, suggesting either that E2F-4 does not require the action of cdk3 bound to DP-1 to fully *trans*-activate a promoter or that DP-1 is not a genuine heterodimeric partner of E2F-4.

E2F activation is an event that appears to be necessary for the transition from the G₁- to the S-phase of the cell cycle (Nevins 1992; Lam and La Thangue 1994; Duronio et al. 1995; Qin et al. 1995), and the data reported here strongly suggest that the G₁ arrest induced by dominant-negative cdk3 is, at least in part, a product of E2F inhibition. Because this inhibition is not the result of pRb binding nor of a specific, negative effect on E2F DNA-binding activity, it seems likely that cdk3 function is required for the activation of E2F as a transcription modulation element, perhaps by allowing specific, functional cross-talk between E2F and either the basal transcription machinery or a putative coactivator required for E2F transcriptional activity. It is also possible that cdk3 contributes to additional steps required for S-phase entry beyond the activation of certain members of the E2F family of transcription factors.

Materials and methods

Cell cultures, transfections, and plasmids

U2OS and VA13 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Fetal-

Clone I; HyClone) at 37°C in a 10% CO₂-containing atmosphere. Cells were transfected by the calcium-phosphate method as described (Chen and Okayama 1987).

The following plasmids were used for transfections: pCMV-*neo*-Bam, pCMV-cdk2wt-HA, pCMV-cdk2dn-HA, pCMV-cdk3wt-HA, pCMV-cdk3dn-HA (van den Heuvel and Harlow 1993); 3xwt-E2F-Luc, 3xmut-E2F-Luc (Krek et al. 1993); pRcCMV-HA-E2F-1, pRcCMV-HA-E2F-1-Δ24, pRcCMV-T7-DP-1 (Krek et al. 1994); pSG5-T, pSG5-K1 (Zalvide and DeCaprio 1995); pSG5-E2F-1 (Qin et al. 1995). pRcCMV-HA-E2F-1-VP16, a generous gift of W. Krek, was constructed by fusing the cDNAs of an E2F-1 *trans*-activation deletion mutant E2F-1-(1-368) with the VP16 *trans*-activation domain (W. Krek and D.M. Livingston, unpubl.). pCMV-CD19 was kindly provided by T. Tedder (Duke University, Durham, NC). pCMV-Puro was constructed using the backbone of pCMV-*neo*-Bam, digested with *Hind*III and *Xba*I, to excise the *neo*-resistance gene, and subsequently treated with Klenow polymerase to generate blunt ends. A SV40-driven puro-resistance gene was excised from pBabe-puro using *Sall* and *Clal*. Blunt ends were generated by treatment with Klenow polymerase, and the fragment was ligated into the pCMV backbone lacking the *neo*-resistance gene. HA-pRbΔp34-SV40-poly(A) was cloned from pECE-HA-Δp34 (Hamel et al. 1990) as an *Eco*RI-*Bam*HI fragment into pCMV-puro, by ligating it to the *Bam*HI-*Sall* and the *Sall*-*Eco*RI fragments obtained from pCMV-puro, in a three fragment ligation, to create pCMV-puro-HA-pRbΔp34.

Cell cycle analysis

Cells were collected by trypsinization 48 hr after removal of the transfection precipitate, washed twice with PBS, 10 mM HEPES (pH 7.3), 0.1% of BSA, and incubated at 4°C for 1 hr in 100 μl of the same buffer with, first a CD19-specific monoclonal antibody (a kind gift of J. Gribben, Dana-Farber Cancer Institute, Boston, MA), and then, after washing twice, with a FITC-conjugated anti-mouse secondary antibody (Boehringer Mannheim). Cells were washed with PBS, 10 mM HEPES (pH 7.3), and fixed in 70% ethanol overnight at 4°C. Before cell cycle analysis, cells were washed with PBS, 10 mM HEPES (pH 7.3), resuspended in 300 μl of 69 μM propidium iodide in 38 mM sodium citrate, containing 5 μg/ml of RNase, and incubated for 30 min at 37°C. Cell cycle analysis was performed with a Becton Dickinson FACScan using SOBR cell cycle analysis software.

Reporter assay

Cells were harvested by trypsinization 24 hr after removal of the transfection precipitate and washed with PBS. To prepare extracts, cells were resuspended in 200 μl of 100 mM KH₂PO₄ (pH 7.8) and three rounds of freeze-and-thawing were performed. The extract was cleared by centrifugation, 10 min at 12,000g, and used for luciferase and β-galactosidase assays, as described (Ausubel et al. 1987).

EMSA

Extracts for EMSA were prepared 24 hr after transfection, as described previously (Krek et al. 1993). The assay was performed as described (Shirodkar et al. 1992) using 10 μg of cell extract and 1 ng of a double-stranded, ³²P-labeled oligonucleotide (5'-CCCCACTGCAATTTCGCGCCAACTTGGG-3') containing an E2F-binding site (underlined) derived from the DHFR promoter (Wade et al. 1992). The E2F-DNA-binding complexes were characterized either by DNA-binding competition, adding 100 ng of cold wild-type oligonucleotide or 100 ng of cold olig-

onucleotide containing a mutated E2F site (TTTCGATC), or by the addition of specific previously characterized antibodies, 0.5 µg of affinity-purified anti-HA antibody (12CA5; Boehringer Mannheim) (Krek et al. 1993), or 2 µl of anti-pRb monoclonal antibody (C36) (Shirodkar et al. 1992).

Western blot analysis

The accumulation of transfected gene products was monitored by Western blotting. Aliquots of cells harvested for cell cycle analysis, reporter assay, or EMSA, were lysed in sample buffer and boiled for 5 min. Proteins were separated in SDS-polyacrylamide gels and transferred to nitrocellulose by standard procedures. Accumulation of HA-tagged gene products and T was monitored by blotting with a monoclonal anti-HA antibody (12CA5; Boehringer Mannheim) and with a monoclonal anti-T antibody (PAB419), respectively. Detection was performed, after incubation with horseradish peroxidase-conjugated sheep-anti-mouse-IgG (Amersham), by enhanced chemiluminescence, according to manufacturer's instructions (Amersham).

Metabolic labeling and immunoprecipitations

Cells were incubated, 24 hr after removal of the transfection precipitate, in 5 ml of methionine-free DMEM (GIBCO), supplemented with 10% dialyzed fetal bovine serum (GIBCO) for 30 min. Then, 0.5 mCi of [³⁵S]methionine (NEN) was added per 10-cm dish and the culture was incubated at 37°C. Three hours later, the monolayer was washed twice with PBS, and the cells were harvested by scraping in 1 ml of TNN buffer [50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM sodium ortho-vanadate, 1 mM DTT, 1 mM PMSE, 20 µg/ml of aprotinin] and incubated for 30 min on ice. For immunoprecipitations using the anti-HA antibody (12CA5), a TNN buffer containing 250 mM NaCl was used to prepare the extracts. Cell extracts were centrifuged for 5 min at 12,000g and 4°C. The supernatants were precleared for 45 min at 4°C on a rocker with 50 µl of a 50% (vol/vol) protein A-Sepharose suspension in TNN buffer. Immunoprecipitations were then performed for 1 hr at 4°C on a rocker with the indicated antibodies: anti-E2F-1 monoclonal antibody SQ41 (Kaelin et al. 1992), anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim), anti-cdk3 polyclonal antibody, Y-20 (Santa Cruz Biotechnology), and anti-T7 monoclonal antibody (Novagen). For the anti-E2F-1 immunoprecipitation with the SQ41 antibody, a rabbit anti-mouse-IgG secondary antibody (Cappel) was added after 30 min of incubation with first antibody. Protein A-Sepharose (50 µl) was added, and the incubation was continued for 45 min. Immunocomplexes were harvested by centrifugation, washed four times with 1 ml of TNN buffer, resuspended in SDS sample buffer, boiled, and resolved by SDS-PAGE. For reimmunoprecipitations, after washing with TNN buffer, the immunocomplexes were resuspended in 100 µl of release buffer [50 mM Tris-HCl (pH 7.4), 1% SDS, 5 mM DTT] and boiled for 10 min. Twenty microliters was analyzed and 80 µl was diluted with 1.4 ml of TNN buffer, subjected to a second immunoprecipitation overnight at 4°C on a rocker, and then processed as described above. For immunoprecipitations performed with native or denatured extracts, metabolically labeled cells were harvested by trypsinization, washed twice with PBS, and resuspended in either 1 ml of TNN buffer (native) or 100 µl of release buffer (denatured). The latter was boiled for 10 min and diluted with 1.4 ml of TNN buffer. Immunoprecipitation was then performed as described above.

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