Residues phosphorylated by TFIIH are required for E2F-1 degradation during S-phase

Laurence Vandel and Tony Kouzarides¹

Wellcome/CRC Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK

¹Corresponding author
e-mail: tk106@mole.bio.cam.ac.uk

The transcription factor E2F-1 plays a key role in regulating cell cycle progression. Accordingly, E2F-1 activity is itself tightly controlled by a series of transcriptional and post-transcriptional events. Here we show that the E2F-1 activation domain interacts with a kinase activity which phosphorylates two sites, Ser403 and Thr433, within the activation domain. We demonstrate that TFIIH is responsible for the E2F-1 phosphorylation observed in cell extracts and that endogenous E2F-1 interacts *in vivo* with p62, a component of TFIIH, during S phase. When the two phosphorylation sites in E2F-1 are mutated to alanine, the stability of the E2F-1 activation domain is greatly increased. These results suggest that TFIIH-mediated phosphorylation of E2F-1 plays a role in triggering E2F-1 degradation during S phase.

Keywords: cdk7/degradation/E2F-1/phosphorylation/TFIIH

Introduction

The E2F family of cellular transcription factors is involved in regulating cell cycle progression, at least in part by controlling the expression of genes which regulate cell proliferative responses: E2F-1-binding sites have been found in the promoters of several regulators of the cell cycle, such as *cdc2*, cyclin E, cyclin A and E2F-1 itself (Dalton, 1992; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Ohtani *et al.*, 1995; Schulze *et al.*, 1995; Geng *et al.*, 1996), and also in the promoters of some proto-oncogenes involved in cell proliferation, including *c-Myb*, *B-Myb*, *c-Myc* and *N-Myc* (Hiebert *et al.*, 1989; Mudryj *et al.*, 1990; Hiebert *et al.*, 1991; Lam and Watson, 1993; Amati *et al.*, 1998). Forced expression of E2F-1 induces quiescent cells to enter S phase, and this activity correlates with the ability of E2F-1 to activate transcription (Johnson *et al.*, 1993). The activity of E2F-1 is regulated at different levels. First, E2F-1, which is absent in quiescent cells, is transcriptionally induced in late G₁ following mitogenic stimuli (Slansky *et al.*, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Ohtani *et al.*, 1995). Secondly, the activity of E2F-1 is regulated through association with other proteins. E2F-1 has an acidic activation domain (AD) located near its C-terminus which has been shown to interact with a number of proteins that regulate its activity either negatively or positively. The tumour suppressor protein p53 (O'Connor *et al.*, 1995) and the retinoblastoma

protein RB inhibit E2F-1 transcriptional activity (Hiebert *et al.*, 1992; Hagemeier *et al.*, 1993; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998), whereas some components of the basal transcriptional machinery, such as TATA box-binding protein (TBP; Hagemeier *et al.*, 1993; Pearson and Greenblatt, 1997), as well as the coactivator CBP (Trouche and Kouzarides, 1996) and the oncoprotein MDM2 (Martin *et al.*, 1995), increase this activity.

The association of RB with E2F-1 is cell cycle regulated and occurs mainly during G₁ phase (for reviews see Dyson, 1998; Helin, 1998). Cyclin D–cdk4, cyclin D–cdk6 and cyclin E–cdk2 all phosphorylate RB during G₁ progression (Ewen *et al.*, 1993; Kato *et al.*, 1993; Lundberg and Weinberg, 1998). As only the hypophosphorylated form of RB binds E2F, RB phosphorylation by these G₁ cyclin-dependent kinases releases ‘free’, transcriptionally active, E2F-1 (Chellappan *et al.*, 1991; Kato *et al.*, 1993) (for a review see Mitnacht, 1998). Phosphorylation of E2F-1 itself by cyclin A–cdk2, which stably interacts with the N-terminus of the former (Krek *et al.*, 1994), may promote (Peeper *et al.*, 1995) or disrupt (Fagan *et al.*, 1994) RB–E2F-1 complexes. Moreover, the phosphorylation of both E2F-1 and DP-1 subunits by cyclin A–cdk2 inhibits the DNA-binding activity of the dimer (Dymlach *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995).

The activity of the E2Fs is also regulated by degradation via ubiquitin-dependent proteolysis *in vivo*. The conjugation of ubiquitin to proteins depends on three well-defined sequential enzymatic reactions, the last one involving the recognition of the substrate by the Skp1-Cullin-F-box protein ligase (SCF), which in turn triggers ubiquitination of the bound substrate and subsequently its degradation by the 26S proteasome. Interestingly, phosphorylation on a specific residue of the substrate can represent the signal of recognition for the SCF complex (for recent reviews see Krek, 1998; Patton *et al.*, 1998). The region of E2F-1 which targets it for degradation has been mapped to its transactivation domain. Interestingly, E2F-1 is stabilized by interaction with RB (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996; Campanero and Flemington, 1997). However, little is known about the molecular mechanisms involved in this process, and several interesting questions remain to be answered: what are the proteins involved in the signalling of E2F-1 degradation? Which residue(s) of the E2F-1 (AD) target(s) E2F-1 for degradation? At which phase of the cell cycle does this process take place?

Interestingly, it has recently been shown that the E2F-1 (AD) also binds to the general transcription factor TFIIH. *In vitro*, TFIIH subunits p62 and p89 bind to E2F-1 (AD) at the RB-binding site (amino acids 409–415), and the interaction between p62 and E2F-1 is disrupted by RB (Pearson and Greenblatt, 1997). TFIIH is a multisubunit

protein complex consisting of nine subunits and several of those functions have been characterized, including helicase/ATPase activity, DNA repair, DNA-binding and kinase activity (for a review see Svejstrup *et al.*, 1996). The kinase activity associated with TFIIF requires three of its subunits, cdk7, cyclin H and MAT-1, which can form a ternary complex. This complex is found either as an independent complex (called free CAK) or as part of the TFIIF complex (Roy *et al.*, 1994; Shiekhatair *et al.*, 1995; Adamczewski *et al.*, 1996). Free CAK and CAK associated with the core TFIIF complex display different substrate specificity. For example, TFIIF(rap74) and TFI-IE(p56) are phosphorylated by TFIIF but not by CAK. Histone H1 and the C-terminal domain of RNA polymerase II (RNA polII CTD) are a better substrate for TFIIF than for CAK, but cdk2 is better phosphorylated by CAK than by TFIIF (Rossignol *et al.*, 1997; Yankulov and Bentley, 1997). However, unlike many other cdks, cdk7, alone or as part of TFIIF, was found to be active throughout most of the cell cycle (Poon *et al.*, 1993; Brown *et al.*, 1994; Matsuoka *et al.*, 1994; Tassan *et al.*, 1994), except in mitosis where TFIIF-associated kinase and transcription activities are inhibited (Akoulitchiev and Reinberg, 1998).

Given that phosphorylation has an important role in the regulation of many transcription factors, we examined whether E2F-1 (AD) could interact with a kinase and be phosphorylated in cell extracts during the cell cycle. Here we present evidence that E2F-1 (AD) interacts with a kinase and is phosphorylated on two sites within this same region in cell extracts. We also show that purified TFIIF, but not CAK, can phosphorylate E2F-1 (AD) and that the major E2F-1 (AD) kinase activity in cell extracts corresponds to TFIIF. We find that the E2F-1 (AD) phosphorylation in cell extracts is cdk7 dependent, and that the recruitment of cdk7 to E2F-1 requires an intact RB-binding site *in vitro*. We also show that the E2F-1 (AD) kinase activity in cell extracts is reduced during G₁ and is inhibited by RB *in vitro*. Moreover, we showed that the p62 subunit of the TFIIF complex associates specifically with E2F-1 (AD) in S phase *in vivo*. Finally, we found that mutation of the two TFIIF phosphorylation sites to non-phosphorylatable residues stabilizes E2F-1. These data suggest that E2F-1 is phosphorylated by TFIIF kinase in S phase and that phosphorylation may trigger its rapid degradation.

Results

The E2F-1 activation domain binds a kinase activity and is phosphorylated by it

To test whether the E2F-1 (AD) (amino acids 380–437) was phosphorylated in HeLa whole-cell extracts, bacterially expressed GST–E2F-1 (AD) wild-type and GST–E2F-1 (AD) proteins, mutated at two putative phosphorylation sites for cdk7, Ser403/Pro404 and/or Thr433/Pro434 (Figure 1A), were used in a pull-down assay in the presence of HeLa whole-cell extracts. After extensive washes, radiolabelled ATP was added to the pull-downs and phosphorylation of the E2F-1 (AD) proteins was examined (Figure 1B). Whereas wild-type E2F-1 (AD) was phosphorylated efficiently, proteins mutated at Thr433 and Pro434 (mutations TP→AA)

or at Ser403 and Pro404 (mutations SP→AA) showed impaired phosphorylation. Moreover, the double mutations (SP/TP→AA/AA) at both sites described above abolished E2F-1 phosphorylation (Figure 1B). Two GST–E2F-1 (AD) proteins, mutated for the binding of RB on residues Tyr411/Phe413 or deleted for amino acids 406–415 (Figure 1A), were also tested in the same *in vitro* kinase assay. Surprisingly, the GST–E2F-1 (AD) mutated on Tyr411 and Phe413 (mutations YF→AA) and the GST–E2F-1 (AD) deleted for the RB-binding site (mutation del 406–415) were not phosphorylated either (Figure 1B). The control of GST protein alone was not phosphorylated in this assay (data not shown). In order to rule out the possibility that every mutation within the E2F-1 activation domain could decrease its phosphorylation, different mutations were introduced into GST–E2F-1 (AD) (Asp390 and Phe391, Glu398 and Phe399, His406 and Glu407, Glu416/417/419, Asp428 and Phe429, all to Ala) which were then tested in the same kinase assay. In this experiment, none of these mutations showed any effect on E2F-1, as for the GST–E2F-1 (AD) mutated on Glu416/417/419 (mutations EEE→AAA) (Figure 1A and B). The results of these experiments showed that E2F-1 (AD) could bind a kinase activity which could phosphorylate this same region of E2F-1.

Purified TFIIF but not purified CAK phosphorylates the E2F-1 activation domain *in vitro*

As TFIIF has been shown to bind to E2F-1 at the RB-binding site via its subunit p62 *in vitro* (Pearson and Greenblatt, 1997), we decided to investigate whether TFIIF kinase activity was responsible for E2F-1 phosphorylation in cell extracts. GST alone, GST–E2F-1 (AD) and purified histone H1 were used in an *in vitro* kinase assay in the presence of either purified TFIIF or purified CAK (gift from J.-M.Egly) which were prepared from HeLa whole-cell extracts fractionated on heparin Ultrogel, Sulfopropyl and DEAE columns (Gerard *et al.*, 1991). Whereas TFIIF phosphorylated the E2F-1 (AD), CAK was not able to do so (Figure 2A). In contrast, histone H1 was phosphorylated by purified CAK and very efficiently by purified TFIIF. GST alone was not phosphorylated by any of these kinases. To confirm the specificity of E2F-1 (AD) phosphorylation by TFIIF, we tested another portion of the protein from amino acid 300 to 380 encompassing several sites of phosphorylation involved in the RB binding for its phosphorylation by purified TFIIF. No detectable phosphorylation could be observed, suggesting a specificity for E2F-1 (AD) recognition of TFIIF (data not shown).

Purified TFIIF phosphorylates Ser403 and Thr433 of E2F-1 (AD)

In order to identify the sites phosphorylated by TFIIF, the wild-type E2F-1 (AD) and the mutated proteins TP→AA, SP→AA, SP/TP→AA/AA and YF→AA (Figure 1A) were used in an *in vitro* kinase assay in the presence of purified TFIIF (Figure 2B). Interestingly, the pattern of phosphorylation of E2F-1 by this kinase was similar to that observed in HeLa cell extracts (compare Figures 2B and 1B). In addition, the protein mutated for RB binding, YF→AA, showed a great reduction of phosphorylation. This observation may suggest a role for this region in the

Fig. 1. The E2F-1 AD can interact with a kinase activity in HeLa cell extracts and E2F-1 is phosphorylated by this kinase. **(A)** Schematic representation of the E2F-1 (AD) sequence. The binding sites for RB and for the TFIH subunit, p62, are shown. The amino acids mutated and deleted within this region are indicated, as well as the name of the corresponding GST fusion protein. **(B)** The same amounts (500 ng) of the wild-type and the mutated GST-E2F-1 (AD) fusion proteins, as indicated in (A), were used in pull-downs from HeLa cell extracts and *in vitro* kinase assays were performed. Reaction products were resolved on a 12% SDS-polyacrylamide gel and phosphorylated proteins were visualized by autoradiography.

binding of TFIH, as described previously (Pearson and Greenblatt, 1997), rather than in the phosphorylation of the Tyr residue which is not a target for the cdk7 Ser/Thr kinase. Moreover, the residues Ser403/Pro404 and Thr433/Pro434 have been shown not to be involved in the interaction of TFIH with E2F-1 (Pearson and Greenblatt, 1997). Thus, these data indicate that both Ser403 and Thr433 are phosphorylated by TFIH *in vitro* and that the RB-binding site is necessary for this phosphorylation.

The cdk7 component of TFIH binds the RB-binding site of E2F-1

To determine if the cdk7 subunit of TFIH could possibly be the kinase which phosphorylated E2F-1 in HeLa cell extracts, we performed pull-downs using the wild-type and the mutated GST-E2F-1 fusion proteins described in Figure 1A, and a histidine-tagged wild-type E2F-1 (amino acids 380–437). After extensive washings, the association of E2F-1 fusion proteins with cdk7 was tested by Western blot analysis (Figure 3A). Cdk7 was found to associate with wild-type E2F-1 (AD) expressed either as a GST or as a His-tag fusion protein. Mutated proteins TP→AA and SP→AA showed no significant decrease of cdk7 binding compared with the wild-type. However, the double point-mutated protein YF→AA and the deleted protein del 406–415, both unable to bind RB, showed a significant decrease in the amount of associated cdk7 (Figure 3A). These data, together with the data described in Figure 1B, suggest that cdk7 binds to the E2F-1 (AD) at the RB-binding site and phosphorylates E2F-1 on Ser403 and Thr433 in HeLa cell extracts.

DRB inhibits the phosphorylation of the E2F-1 activation domain *in vitro*

Further evidence that TFIH might be the kinase which phosphorylates the E2F-1 (AD) was obtained by the use of DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), a potent inhibitor of cdk7 (Yankulov *et al.*, 1995). HeLa cell extracts were subjected to the E2F-1 (AD) pull-down kinase assays as described, and the effect of DRB on E2F-1 (AD) phosphorylation was examined. DRB inhibited E2F phosphorylation to the same extent as it previously was shown to inhibit phosphorylation of GST-RNA pol II CTD and GST-cdk2 by TFIH (Yankulov *et al.*, 1995). As a control, GST-cJun delta region (amino acids 30–79) (a kind gift from A.Bannister) was used in a pull-down experiment using HeLa cells irradiated with UV in order to induce Jun kinases. *In vitro* kinase assays in the absence or in the presence of 200 or 500 μM DRB showed no change in cJun phosphorylation, ruling out the possibility of a general non-specific inhibition of E2F-1 (AD) phosphorylation by DRB. H8 (*N*-[2-(methylamino) ethyl]-5-isoquinoline sulfonamide dihydrochloride), another inhibitor of TFIH kinase activity (Serizawa *et al.*, 1993), was also capable of decreasing E2F-1 phosphorylation very efficiently at concentrations similar to those of DRB (data not shown).

In order to exclude completely a participation of other cdk7s in the phosphorylation of the E2F-1 (AD), we used two compounds, roscovitine and olomoucine, which are potent chemical inhibitors of cdk7s (cdc2, cdk2, cdk5 and, to a lesser extent, cdk6) (Meijer, 1995). No effect on E2F phosphorylation was observed when these compounds

Fig. 2. Purified TFIH, but not purified CAK, phosphorylates the E2F-1 (AD) on Ser403 and Thr433. (A) *In vitro* kinase assays with the GST protein alone, GST-E2F-1 (AD) and histone H1 were performed in the presence of purified TFIH or purified CAK. The reaction products were resolved on a 12% SDS-polyacrylamide gel and the phosphorylated proteins were visualized by autoradiography. (B) *In vitro* kinase assays were performed with the mutated GST-E2F-1 (AD) fusion proteins as described in Figure 1A, or with histone H1, in the presence of purified TFIH. The reaction products were analysed as described in (A).

were added at a wide range of concentrations (from 0 to 1 mM) to the kinase reactions (data not shown).

Depletion of cdk7 from HeLa cell extracts decreases E2F-1 phosphorylation

To strengthen the notion that cdk7, the kinase subunit of TFIH, can phosphorylate E2F-1, we decided to test whether depletion of cdk7 from cell extracts affected the phosphorylation of E2F-1. HeLa cell extracts which had been subject to cdk7 immunodepletion were used in a GST-E2F-1 (AD) pull-down followed by a kinase assay. As a control, an antibody raised against the DNA-binding domain of Gal4 replaced the cdk7 antibody. In this experiment, E2F-1 phosphorylation was significantly reduced (~70%) in the extracts depleted with the anti-cdk7 antibody compared with those extracts depleted with the anti-Gal4 antibody (Figure 4A). The presence of cdk7 in the extracts was monitored in parallel by Western blot analysis to confirm the efficiency of the immunodepletion. No detectable cdk7 was present in the supernatant of the

Degradation of E2F-1 by phosphorylation

Fig. 3. (A) Cdk7 interacts with E2F-1 (AD) at the RB-binding site. Pull-downs from HeLa cell extracts were performed with the GST protein alone, the wild-type and the mutated GST-E2F-1 (AD) as described in Figure 1A, and with the His-E2F-1 (AD). After extensive washes, the proteins bound to the various fusion proteins were resolved on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose and immunoblotted with the anti-cdk7 antibody. (B) DRB inhibits the phosphorylation of the E2F-1 (AD). Pull-downs with the wild-type GST-E2F-1 (AD) from HeLa cell extracts or with the GST-c-Jun (30-79) from UV-irradiated HeLa cells (120 J/m² for 30 min) were performed, followed by the kinase reactions in the absence or presence of an increasing amount of DRB. Phosphorylated products were loaded on a 12% SDS-polyacrylamide gel and the relative phosphorylation of GST-E2F-1 or GST-c-Jun (31-79) was quantified on a phosphorimager and plotted as dose-response curves for DRB; 100% phosphorylation was set for the phosphorylation of GST-E2F-1 (AD) or of GST-c-Jun (30-79) in the absence of DRB.

cdk7-depleted extracts after immunoprecipitation and Western blot analysis (Figure 4B).

Expression of a dominant-negative cdk7 reduces phosphorylation of E2F-1

In order to demonstrate further the involvement of TFIH in E2F-1 (AD) phosphorylation observed in cell extracts, we used a cdk7 dominant-negative (DN) expression vector encoding an inactive version of the cdk7 kinase. Extracts from cells expressing cdk7 DN, E2F-1 phosphorylation was decreased (~40%) compared with the phosphorylation observed in the extracts from cells expressing empty vector (Figure 4C). The expression level of cdk7 DN was checked by Western blot analysis and was ~2-fold higher than the level of endogenous cdk7 (data not shown).

E2F-1-interacting kinase activity phosphorylates several known TFIH substrates

Next, we tested whether the E2F-1 (AD)-interacting kinase could phosphorylate substrates previously reported to be phosphorylated efficiently by TFIH. His-E2F-1 (AD) was used to pull-down kinase activity from HeLa cell extracts. This activity was eluted from the beads, and added to

different substrates in a kinase reaction. As a positive control, His-E2F-1 (AD) precipitated a kinase which phosphorylated His-E2F-1 itself efficiently (Figure 4D, right panel). GST alone and rap30 of purified TFIIIF, which are not substrates for TFIIH kinase, were not phosphorylated by the E2F-1 (AD)-bound kinase, whereas RNA pol II CTD, cdk2, the rap74 subunit of purified TFIIIF and histone H1 were phosphorylated (Figure 4D, right panel). Moreover, when the kinase activity pulled down with His-E2F-1 (AD) was checked by Western blot analysis for the presence of p62, p89 and cdk7, the three subunits of TFIIH were found to be associated with His-E2F-1 (AD) (Figure 4D, left panel). Thus, these results strongly suggest that TFIIH kinase activity is responsible of E2F-1 (AD) phosphorylation *in vitro*.

E2F-1-associated kinase activity is high in S phase and low in G₁

We next investigated whether E2F-1 kinase activity changed with the cell cycle phase. HeLa cells were

synchronized in different phases of the cell cycle, and extracts prepared from these cells were assessed for their kinase activity towards the E2F-1 (AD) in a pull-down assay. Synchronized extracts in S and G₂ phase showed an equal and high kinase activity against E2F-1 (AD) compared with mid- and especially late G₁ extracts (Figure 5A). As cdk7 activity has been shown not to change during the G₁, S and G₂ phases of the cell cycle (Tassan *et al.*, 1994) and as we have mapped the binding site of cdk7 to E2F-1 (AD) at the RB-binding site, these data suggest that the decrease in phosphorylation of E2F-1 in G₁ could be due to the presence of RB bound to E2F-1 during this phase, thus inhibiting the binding of TFIIH and subsequently the phosphorylation of E2F-1 (AD).

RB inhibits E2F-1 phosphorylation *in vitro*

In order to confirm the above hypothesis, bacterially expressed His-RB fusion protein was tested for its capacity to inhibit E2F-1 (AD) phosphorylation. His-RB was mixed with GST-E2F-1 (AD) to allow the relevant complexes to form. The complexes were washed thoroughly before being used in pull-down kinase assays of HeLa cell extracts. A decrease of ~90% in the E2F-1 (AD) phosphorylation was observed in the presence of RB (Figure 5B). These results suggest that *in vitro*, RB binds to E2F-1 (AD) on the same region as TFIIH, as described previously (Pearson and Greenblatt, 1997), and that RB can displace the binding of the kinase.

Fig. 4. E2F-1 phosphorylation is dependent on cdk7. (A) Depletion of cdk7 from HeLa cell extracts decreases E2F-1 phosphorylation. HeLa cell extracts were depleted with either an anti-cdk7 antibody or an anti-Gal4 antibody. The corresponding depleted extracts were used for pull-downs with wild-type GST-E2F-1 (AD) or with GST alone, followed by kinase reactions. The relative phosphorylation of GST-E2F-1 (AD) or of GST alone was quantified on a phosphorimager and plotted; 100% phosphorylation was set for E2F-1 phosphorylation by HeLa cell extracts depleted with the anti-Gal4 antibody. (B) No cdk7 is detectable in HeLa cell extracts immunodepleted with an anti-cdk7 antibody. HeLa cell extracts not depleted or depleted with an anti-Gal4 antibody or an anti-cdk7 antibody were resolved on a 12% SDS-polyacrylamide gel and tested for the presence of cdk7 by Western blot analysis using an anti-cdk7 antibody. (C) Expression of a dominant-negative form of cdk7 in 293T cells reduces E2F-1 phosphorylation in these extracts. pcDNA3 cdk7 DN (denoted pcD3 cdk7 DN), coding for a dominant-negative form of cdk7, or the control pcDNA3 (denoted pcD3) were transfected into 293T cells and, 48 h later, cells extracts were assessed for the kinase activity associated with E2F-1 by using standard pull-down and kinase assays. Relative E2F-1 phosphorylation was quantified on a phosphorimager and plotted; 100% phosphorylation was set for E2F-1 (AD) phosphorylation by HeLa cell extracts expressing the control vector pcDNA3. (D) The E2F-1 (AD)-bound kinase phosphorylates four substrates of TFIIH and the bound proteins comprise p62, p89 and cdk7. Right panel: His-E2F-1 (AD) was used to pull down the kinase activity from HeLa cell extracts. This activity was eluted from the nickel-agarose beads with imidazole and was added to the different substrates for TFIIH kinase such as RNA polII CTD (GST-CTD), cdk2, purified TFIIIF (rap74 and rap30) and histone H1. GST alone and rap30 of purified TFIIIF served as negative controls. The kinase reaction products were loaded on a 12% SDS-polyacrylamide gel and phosphorylated proteins were visualized by autoradiography. The arrow shows the band corresponding to the phosphorylated His-E2F-1 (AD). Asterisks indicate the positions of the different substrates on the gel. Left panel: in parallel with the kinase reactions, the proteins bound to E2F-1 (AD) were resolved on a 10% SDS-polyacrylamide gel, and Western blot analyses were performed using anti-p62, anti-p89 and anti-cdk7 antibodies.

Fig. 5. (A) E2F-1 kinase activity in cell extracts is decreased during the G₁ phase of the cell cycle. The same amounts of cell extracts from HeLa cells synchronized in the different phases of the cell cycle were used in a pull-down with GST-E2F-1 (AD) followed by a kinase assay. The reaction products were resolved on a 12% SDS-polyacrylamide gel and E2F-1 phosphorylation was quantified on a phosphoimager. Relative E2F-1 phosphorylation in the various cell cycle phases was plotted; 100% phosphorylation was set arbitrarily to the level of phosphorylation observed in G₂. (B) RB inhibits E2F-1 phosphorylation *in vitro*. Different amounts (0–500 ng) of eluted His-RB fusion protein were mixed with 500 ng of GST-E2F-1 (AD) to allow E2F-RB complexes to form. The complexes were washed thoroughly before being used for pull-downs in HeLa cell extracts followed by kinase assays. Reaction mixtures were run on a 12% SDS-polyacrylamide gel and phosphorylation of the E2F-1 (AD) was quantified on a phosphoimager. Relative phosphorylation was plotted and 100% was set for E2F-1 phosphorylation in the absence of RB.

Endogenous p62 and E2F-1 interact during S phase *in vivo*

The p62 subunit of TFIIF was shown previously to interact with E2F-1 *in vitro* (Pearson and Greenblatt, 1997). We decided to determine whether p62 and E2F-1 also interact *in vivo*. Western blots of HeLa cell extracts synchronized in early G₁, late G₁, early S and G₂ were analysed by Western blot for the expression levels of endogenous E2F-1 and p62. Endogenous E2F-1 expression was very weak in early G₁, then peaked in late G₁/early S phase (Figure 6A) and started to decrease from mid- to late S phase (data not shown), returning to a low level in G₂ (Figure 6A). In contrast, in the same experiment using an anti-p62 antibody, the p62 expression level was constant throughout the cell cycle (Figure 6B). Immunoprecipitations of endogenous E2F-1 followed by immunodetection with an anti-E2F-1 antibody showed the same pattern of expression as that described above (compare Figure 6C with A). This result confirmed that E2F-1 immunoprecipitates throughout the cell cycle were quantitative. When endogenous E2F-1 immunoprecipitates were immunoblotted for the presence of p62, only extracts from cells synchronized in early S phase showed a significant amount of associated p62 (Figure 6D). These results

Degradation of E2F-1 by phosphorylation

Fig. 6. Endogenous p62 and E2F-1 interact in S phase *in vivo*. (A) HeLa cell extracts synchronized in early G₁, late G₁, early S and G₂ were resolved on a 10% SDS-polyacrylamide gel and were tested for the endogenous expression level of E2F-1 by Western blot analysis using the anti-E2F-1 monoclonal antibody KH95. (B) The same blot as described in (A) was stripped and immunoblotted with the anti-p62 monoclonal antibody to detect the p62 endogenous expression level. (C) Immunoprecipitations (IP) of endogenous E2F-1 with the anti-E2F-1 polyclonal antibody C20 were performed in HeLa cell extracts synchronized in the various cell cycle phases as indicated. The precipitated proteins were loaded onto a 10% SDS-polyacrylamide gel and were immunoblotted with the anti-E2F-1 monoclonal antibody KH95 to detect the E2F-1 expression level. (D) The same blot as described in (C) was stripped and immunoblotted with the monoclonal anti-p62 antibody to detect the p62 protein co-immunoprecipitated with E2F-1 during the cell cycle. The intensity of each band obtained for all the Western blots was quantified after scanning with the NIH image software. The corresponding values were plotted underneath each Western blot as a graph; 100% was set for each Western blot for the phase of the cell cycle where the maximal intensity was obtained.

indicate that p62 specifically interacts with E2F-1 in S phase *in vivo*.

RB inhibits the transcriptional activity of mutated forms of E2F-1 (AD)

RB binds to E2F-1 (AD) and represses E2F-1 transcriptional activity. As we have shown, RB also inhibits E2F-1 (AD) phosphorylation (Figure 5B). Thus, we decided to analyse whether E2F-1 phosphorylation played a role in E2F-1 transcriptional activity and whether this phosphorylation was important for RB-mediated repression of transactivation. E2F-1 (AD) mutants Ser403 or/and Thr433 were mutated either to Ala (a non-phosphorylatable residue) or to Asp (a residue which can sometimes mimic phosphorylated Ser or Thr) and they were fused to the DNA-binding domain of Gal4 (Figure 7A). The resulting fusion proteins were expressed in 293T cells and their transcriptional activity was assessed in the absence or presence of RB. As shown in Figure 7B, all fusion proteins examined activated the transcription of a gene downstream of a promoter which contained five binding sites for Gal4. Moreover, RB could repress with the same efficiency the

Fig. 7. The non-phosphorylatable form of the E2F-1 (AD) shows greatly increased stability *in vivo*. (A) Schematic representation of the sequence of the E2F-1 (AD). The p62- and the RB-binding sites are shown. The amino acids mutated either into Ala or into Asp within this region are indicated, as well as the name and the number of the corresponding Gal4 DNA-binding domain fusion protein. (B) Mutations of Ser403 and Thr433 into Ala or Asp do not play a major role in E2F-1 transcriptional activity. A 1 μ g aliquot of each construct pHKGal4-E2F-1 (AD), as described in (A), and numbered from 1 to 7, was transfected into 293T cells with 1 μ g of reporter plasmid pBS 5 $\times$ Gal4-M2-luc in combination with either 0.5 μ g of the empty vector pCMV (-) or with 0.5 μ g of pCMV-RB (+). After 48 h, the transcriptional activity of each protein was assessed by luciferase assay and the values were plotted in arbitrary units. (C) For the protein stability experiment, 5 μ g of wild-type or point-mutated pHKGal4-E2F-1 (AD) constructs as described in (A) were transfected into 293T cells. After 40 h, DMEM containing 25 μ g/ml of cycloheximide was added to the cells (time 0). Cells were harvested every 30 min during 2 h and cell extracts were loaded on a 12% SDS-polyacrylamide gel. The protein level of the wild-type and various mutated Gal4-E2F-1 (AD) at the different times was assessed by Western blot analysis using the anti-E2F-1 polyclonal antibody C20. The half-life ($t_{1/2}$) of each protein was calculated after scanning and quantification of the bands on the Western blots with the NIH image software. The values obtained were plotted on a graph shown in (D). (D) A value of 100% was set for each construct at time 0. The symbol of the line corresponding to each protein tested for the degradation is indicated to the right of the half-life of each construct in (C).

transactivation induced by any of the E2F-1 proteins tested (Figure 7B). These results suggest that phosphorylation or lack of phosphorylation of E2F-1 (AD) at Ser403 or/and Thr433 does not play a major role either in the transactivation capacity of E2F-1 (AD) or in the repression by RB.

The non-phosphorylatable form of E2F-1 (AD) shows greatly increased stability *in vivo*

Another proposed role for RB is stabilization of E2F-1 by protecting it from degradation (Hateboer *et al.*, 1996; Hofmann and Livingston, 1996; Campanero and Fleming-ton, 1997). Thus, it was interesting to analyse E2F-1 degradation, using the protein synthesis inhibitor cycloheximide. The wild-type and the various Gal4-E2F-1 (AD)

proteins with Ser403 or/and Thr433 mutated to Ala or Asp residues (Figure 7A) were introduced into 293T cells and their expression levels were assessed at different times after cycloheximide addition (Figure 7C). The Ser403 to Ala and the Thr433 to Ala mutated proteins showed a slight increase in their stability compared with the wild type (half-lives of 78, 58 and 43 min, respectively). Interestingly, the double mutated protein, Ser403/Thr433 to Ala, showed a very high stability (half-life >120 min; Figure 7D), suggesting some cooperation between the two sites in E2F-1 stability. The corresponding mutations to Asp did not decrease the stability of the protein significantly (Figure 7C), suggesting that the Asp residue does not mimic phosphorylated Ser or Thr residues in this context. However, our data show that the lack of phospho-

rylation of E2F-1 (AD) at Ser403 and Thr433 correlates with an increase in the stability of E2F-1.

Discussion

It has been shown that E2F-1 is degraded by the ubiquitin-dependent proteolysis pathway and that targeting for degradation requires the E2F-1 AD. Moreover, E2F-1 is stabilized by its interaction with RB (Hateboer *et al.*, 1996; Hofmann and Livingston, 1996; Campanero and Flemington, 1997). However, so far little is known about the signals which trigger E2F-1 degradation. In this study, we have shown that, in cell extracts, E2F-1 (AD) interacts with a kinase activity which phosphorylates this same region. The two sites phosphorylated correspond to typical putative cdk's or MAPK phosphorylation sites. We show that this phosphorylation is carried out by the TFIID kinase and that phosphorylation of E2F-1 results in its degradation.

When we used HeLa cell extracts to look for kinase activities which could bind to E2F-1 (AD), we identified an activity which gave a phosphorylation pattern similar to that observed with purified TFIID kinase *in vitro*, demonstrating that TFIID is probably the major E2F-1 kinase in cell extracts. We identified the two sites phosphorylated within E2F-1 (AD), Ser403 and Thr433, and we demonstrated that cdk7, the kinase subunit of TFIID, could associate with E2F-1 (AD) at the RB-binding site *in vitro*. These results are supported by previous *in vitro* data showing that p62, another component of TFIID, binds to E2F-1 at the RB-binding site and displaces RB (Pearson and Greenblatt, 1997). This finding may explain the fact that purified TFIID, but not purified CAK which lacks the p62 subunit, phosphorylated E2F-1 (AD) *in vitro*. Such a difference in substrate specificity between TFIID and CAK has also been described for the general transcription factors TFIIF (rap74 subunit) and TFIIE (p56 subunit), which are phosphorylated by TFIID but not by free CAK (Yankulov and Bentley, 1997). One of the subunits of TFIID which could play the role of a bridge between E2F-1 (AD) and TFIID is p62, both *in vitro* (Pearson and Greenblatt, 1997) and *in vivo*, as we have shown here. Hence, the decrease of phosphorylation of an E2F-1 (AD) mutated protein which cannot bind RB is probably due to the inability of this protein to interact with p62, and thus to recruit TFIID to the E2F-1 (AD). We can rule out the possibility that the E2F-1 protein mutated for the RB-binding site was misfolded because it could still bind, as efficiently as the wild type, other proteins such as Mdm2 (Martin *et al.*, 1995).

Further evidence supporting the role of TFIID in E2F-1 (AD) phosphorylation was obtained using an inhibitor of TFIID kinase activity. Indeed, the inhibition of E2F phosphorylation by the protein kinase inhibitor DRB showed the same concentration dependence as that described for the phosphorylation of RNA polII CTD by purified TFIID (Yankulov *et al.*, 1995). Moreover, the decrease in E2F phosphorylation by cdk7-depleted cell extracts suggests that cdk7 contributes to at least 70% of the observed phosphorylation. However, even though we could not detect any cdk7 by immunoblot, it is possible that a small amount of cdk7 which could not be detected by Western blot analysis accounted for the remaining

phosphorylation of E2F-1 (AD). The 40% decrease of E2F phosphorylation observed in cell extracts expressing a dominant-negative form of cdk7 also suggests that this protein can inhibit some E2F-1 phosphorylation. However, the remaining kinase activity was still high. As the transfection efficiency was not 100% and as the expression of the dominant-negative form of cdk7 was only 2-fold higher than the expression level of endogenous cdk7, there were probably active endogenous cdk7 molecules which interacted with E2F-1 and phosphorylated it. Finally, specific substrates for TFIID kinase (TFIIF, cdk2 and RNA polII CTD) (Rossignol *et al.*, 1997; Yankulov and Bentley, 1997) were phosphorylated by the kinase which interacted with the E2F-1 (AD) in cell extracts. We showed in parallel that both p62 and p89 (two subunits of the core TFIID) and cdk7 were pulled down with the E2F-1 (AD). Altogether, these results strongly suggest that E2F-1 (AD) is phosphorylated by TFIID kinase *in vitro*. Among the cdk's, only CAK/TFIID kinase has been shown to be constitutively active during G₁, S and G₂ phases (Tassan *et al.*, 1994). As purified TFIID required the RB-binding site on E2F-1 to phosphorylate E2F-1 (AD) and as cdk7 from cell extracts, probably via p62, bound to E2F-1 on the same site, we hypothesized that the lack of phosphorylation of E2F-1 during G₁ was due to RB-mediated inhibition of binding of p62, which recruits the whole TFIID complex (Pearson and Greenblatt, 1997).

Our experiments using synchronized cell extracts to pull down a kinase activity with the E2F-1 (AD) fusion protein did not take into consideration the actual expression level of E2F-1 during the cell cycle. Indeed, our experiments and those of others (Kaelin *et al.*, 1992; Shan *et al.*, 1992) showed a peak of expression at the G₁/S transition followed by a rapid decrease from mid-S to G₂ phase until the next G₁/S transition. Our results from *in vivo* co-immunoprecipitations of endogenous E2F-1 and p62 showed a specific interaction between these two proteins in early S phase compared with during G₁. However, we could not detect any interaction between E2F-1 and p62 during the other phases of the cell cycle. This could be due to the very low level of expression of E2F-1 at these stages.

Interestingly, it has been proposed that the decrease in E2F-1/DP-1 activity in mid- to late S phase depends on the action of cyclin A-cdk2 which binds to the N-terminal region of E2F-1, phosphorylates both E2F-1 and DP-1 and down-regulates the DNA-binding capacity of the heterodimer, probably by destabilizing it (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995). However, the importance of the down-regulation of E2F-1 by phosphorylation by cyclin A-cdk2 in mid- to late S phase, when we observed a very weak expression of E2F-1, is not clear.

As we showed a rapid decrease of E2F-1 expression level during the S phase of the cell cycle, we examined whether phosphorylation of the E2F-1 (AD) had an effect on E2F-1 stability. We observed that the E2F-1 protein where Ser403 and Thr433 were mutated to Ala (mutation S/T→A/A) displayed an enhanced stability compared with the wild-type, indicating that the lack of phosphorylation of the two sites, Ser403 and Thr433, prevents E2F-1 from degradation. The stabilization of the E2F-1 (AD) mutated at Ser403 and Thr433 to Ala was not due to an increase

of RB binding, since RB could repress the transcriptional activity of all the mutants to the same extent (Figure 7B). Because TFIIF binds to E2F-1 specifically in S phase *in vivo* when E2F-1 is degraded rapidly, and because TFIIF phosphorylates the two sites regulating the stability of E2F-1 within its AD *in vitro*, we suggest that the phosphorylation of E2F-1 could trigger its rapid degradation in S phase. The increased stability observed for the E2F-1 protein mutated for the TFIIF phosphorylation sites could be explained by the inability of specific proteins, involved in the targeting of E2F-1 for degradation, to recognize and to bind to this mutated E2F-1. This hypothesis is supported by the fact that the mutations of Ser403 and Thr433 to Asp residues within E2F-1 (AD) did not destabilize it, suggesting either that the Asp residues did not mimic phosphorylated Ser and Thr residues or that phosphorylation of Ser403 and Thr433 residues is required specifically for the degradation of the E2F-1 (AD). Indeed, the phosphorylation of one or two residues of several proteins involved in cell cycle regulation has been shown to target them for degradation. Such is the case for cyclin D1 (Diehl *et al.*, 1997), cyclin E (Clurman *et al.*, 1996; Won and Reed, 1996) and the cdk inhibitor p27^{KIP} (Ellis *et al.*, 1999; Mann *et al.*, 1999; Nguyen *et al.*, 1999). Recently, the highly unstable transcription factor Myc has also been shown to be destroyed by ubiquitin-dependent proteolysis *in vivo*. As for E2F-1, it is the transactivation domain which promotes its degradation. Two mutated forms of Myc have been found in lymphomas which consist of Thr58 which is mutated to an Ala, and of Ser71 which is mutated to a Tyr. These mutations have been shown to be associated with an enhanced stability of the c-Myc protein, which is believed to be responsible of the oncogenic transformation by c-Myc (Salghetti *et al.*, 1999) and, interestingly, Thr58 at least has also been shown to be a major site of Myc phosphorylation *in vitro* and *in vivo* (Lutterbach and Hann, 1994; Pulverer *et al.*, 1994). The kinase involved in this process is still not known.

Further analysis aimed at understanding the fine control of phosphorylation in transformed cells versus non-transformed cells hopefully would provide more evidence on the importance of phosphorylation for E2F-1 activity or of other components, and on its consequences in cell cycle regulation.

Materials and methods

Bacterially expressed proteins

cDNAs of E2F-1 (amino acids 380–437) wild-type and double point mutants to Ala at Ser403 and at Pro404 (mutations SP→AA), Thr433 and Pro434 (mutations TP→AA), Tyr411 and Phe413 (mutations YF→AA), as well as the mutant at Ser403, Pro404, Thr433 and Pro434 (mutations SP/TP→AA/AA), were introduced into pGEX. The corresponding GST fusion proteins were expressed in XA90 bacteria and purified with glutathione–Sepharose beads as described previously (Hagemeier *et al.*, 1993). As controls, we also used five other GST–E2F-1 (AD) proteins mutated at different residues into Ala (Asp390 and Phe391, Glu398 and Phe399, His406 and Glu407, Glu416/417/419, Asp428 and Phe429) which have been expressed and purified as described (Hagemeier *et al.*, 1993). GST–RNA polII CTD was expressed as described previously (Peterson *et al.*, 1992). Wild-type E2F-1 (AD) (amino acids 380–437) cDNA was introduced into the pQE30 vector (Qiagen) in-frame with a 6×His tag at its N-terminus. The resulting fusion protein was expressed in M15[rep4] bacteria as described by the manufacturer (Qiagen). His-RB (379–928) was expressed as described

previously (Brehm *et al.*, 1998), and bound proteins on Ni²⁺-NTA-agarose beads were eluted with 150 mM imidazole in Tris-buffered saline.

In vitro kinase assays

Kinase assays were performed with ~500 ng of GST–E2F (AD) or GST alone and 100 ng of histone H1 (Boehringer Mannheim) in a 30 µl volume of reaction buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), phosphatase inhibitors (1 mM Na₃VO₄, 5 mM NaF), 50 µM ATP and 2.5 µCi of [γ -³²P]ATP. Purified CAK (200 ng) (Rossignol *et al.*, 1997) or highly purified soluble (HAP fraction) TFIIF (50 ng) (a gift from J.-M.Egly) prepared as described in Gerard *et al.* (1991) were then added to the different substrates. Reaction mixtures were incubated at 30°C for 30 min, and the reactions were stopped by the addition of SDS loading buffer. Reaction mixtures were run on a 12% SDS–polyacrylamide gel which was fixed, stained with Coomassie Blue and dried before autoradiography. Quantifications of phosphorylated proteins were performed on a Fuji BAS-2500 Phosphorimager. Gels stained with Coomassie Blue were also scanned by densitometry (Umax Scan) to quantify the exact amount of protein present on the gel by NIH Image software.

Pull-downs of HeLa whole cell extracts and in vitro kinase assays

HeLa whole-cell extracts were prepared in lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, proteases inhibitors [100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 µg/ml leupeptin and 1 µg/ml pepstatin] and phosphatase inhibitors as described above. A 250 µg aliquot of cell extracts and 500 ng of GST fusion proteins were used for each pull-down. After 90 min of incubation on a rotating wheel at 4°C, the beads were washed extensively three times in lysis buffer and twice in reaction buffer. Kinase assays were then performed on beads in a 30 µl reaction volume in the presence of 50 µM ATP and 2.5 µCi of [γ -³²P]ATP as described above. The reaction products were run on a 12% SDS–polyacrylamide gel. Phosphorylation of the GST fusion proteins was quantified on a phosphorimager.

Pull-downs with His-E2F-1 (AD) bound to Ni²⁺-NTA-agarose beads were performed as described for the GST fusion proteins except that HeLa cell extracts were prepared in lysis buffer in the absence of EDTA. This buffer was used for all the pull-downs and the washes. The washes were performed in reaction buffer without any DTT.

His-E2F-1 (AD) and the proteins bound to it were eluted from the beads by three 15 min incubations in reaction buffer containing 1 mM DTT and 150 mM imidazole. The supernatants were collected and pooled before being added (20 µl) to the various substrates in the presence of 50 µM ATP and 2.5 µCi of [γ -³²P]ATP. The amount of substrate used was ~500 ng of GST–E2F-1 (AD) or of GST alone, 50 ng of recombinant rap74, rap30 and the subunits of TFIIF (gift from S.Roberts), 50 ng of His-cdk2 (gift from J.Pines) and 100 ng of histone H1 (Boehringer Mannheim).

A 1 mg aliquot of HeLa cell extracts was used in a pull-down with 4 µg of His-E2F-1 (AD) and, after three washes in lysis buffer, the pulled-down products were resolved on a 10% SDS–polyacrylamide gel followed by a Western blot and immunodetection of p62, p89 and cdk7 using the p62 and p89 monoclonal antibodies (a gift from J.-M.Egly) and the polyclonal anti-cdk7 antibody (UBI).

Increasing amounts (0–500 ng) of His-RB eluted from Ni²⁺-NTA-agarose beads with 150 mM imidazole were mixed with 500 ng of GST–E2F (AD) in Z' buffer (25 mM HEPES pH 7.5, 12.5 mM MgCl₂, 150 mM NaCl, 0.1% NP-40, 20% glycerol) in the presence of 1 mM DTT and 150 µg/ml bovine serum albumin. After 1 h of gentle rocking incubation at room temperature, the reactions were spun down and washed four times with NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). E2F–RB complexes bound to the beads were added to 250 µg of HeLa cell extracts, and pull-downs followed by kinase assays were performed in the presence of phosphatase inhibitors as previously described. The reaction products were resolved on a 12% SDS–polyacrylamide gel and E2F-1 phosphorylation was quantified on a phosphorimager.

Western blots and immunodepletions

Lysates prepared in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40) were used for Western blot according to standard protocols. The proteins were revealed by enhanced chemiluminescence (Amersham). All the autoradiography films were pre-flashed prior to exposure in order to obtain a linear signal.

For each immunoprecipitation, a similar amount of HeLa cell extracts (500 µg) diluted in RIPA buffer was used with 3 µg of either the anti-

cdk7 antibody (UBI) or the control anti-Gal4 antibody (Santa Cruz) in the presence of protein A/G-Sepharose. This immunodepletion was performed three times on the collected supernatant. The depletion was confirmed by Western blot analysis on the pellets and on the supernatants using the anti-cdk7 antibody.

The extracts depleted with the anti-cdk7 or the anti-Gal4 antibody were then tested in *in vitro* kinase assays with GST alone or with GST-E2F-1 (AD) as described before.

Transfections

A 20 µg aliquot of pcDNA3 encoding a dominant-negative form of cdk7 tagged at its C-terminus with a triple Myc epitope (Mäkelä *et al.*, 1995) or 20 µg of pcDNA3 as a control were transfected in 145 mm plates of 293T cells by calcium phosphate co-precipitation. After 48 h, cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer supplemented with proteases inhibitors. Extracts were then tested for the expression of dominant-negative cdk7 by Western blot using the anti-cdk7 antibody (UBI) before using these extracts in kinase assays.

Synchronization of HeLa S3 cells

HeLa S3 cells were cultured as exponentially growing monolayers on 145 mm plates in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (Gibco-BRL), 10 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco-BRL). Cells were synchronized in S phase by a single blocking culture medium containing 2.5 mM thymidine (Sigma) for 24 h (Rao and Johnson, 1970) followed by a release into the culture medium for 1 (early S phase) and 4 h (mid-S phase). Cells were synchronized in G₂ by releasing the thymidine-blocked cells in very early S phase into culture medium for 10 h before proceeding to a second thymidine block for 16 h, followed by a release into a culture medium containing 40 ng/ml nocodazole (Sigma) for ~8 h. Cells in G₁ were obtained by releasing the cells blocked with thymidine into nocodazole-containing culture medium for 12 h to arrest them in mitosis. These mitotic cells were then released into fresh culture medium for 2 (early G₁), 6 (mid-G₁) or 10 h (late G₁). Cell cycle synchronization was verified by flow cytometry of isolated nuclei which were prepared as described previously (Krude *et al.*, 1997).

Similar amounts (250 µg) of HeLa cell extracts synchronized in mid-G₁, late G₁, early S, mid- and G₂ were diluted in lysis buffer and used for pull-downs in the presence of GST-E2F-1 (AD). Kinase assays were performed as described above.

Endogenous co-immunoprecipitations

HeLa whole-cell extracts (6 mg) synchronized in G₁, S and G₂ were diluted in 1.5 ml of RIPA buffer and pre-cleared with 50 µl of protein A/G-Sepharose for 30 min at 4°C. Then 4 µg of the polyclonal anti-E2F-1 antibody C20 (Santa Cruz) or a control anti-Gal4 antibody were added to the extracts and immunoreaction was performed for 2 h at 4°C on a rotating wheel. A 30 µl aliquot of protein A/G-Sepharose was then added for another 2 h at 4°C. After three extensive washes in RIPA buffer, the beads were loaded on a 10% SDS-polyacrylamide gel which was followed by a Western blot and immunodetection with the monoclonal anti-p62 antibody (a generous gift from J.-M.Egly). The blot was stripped for 30 min at 50°C in buffer S (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.7), washed twice for 10 min in PBS, 1% Triton X-100 at room temperature, and reblotted with the monoclonal anti-E2F-1 antibody KH95 (Santa Cruz).

In order to assess the expression levels of the endogenous proteins p62 and E2F-1 throughout the cell cycle, 150 µg of whole-cell extract synchronized in the various cell cycle phases were also loaded on a 10% SDS-polyacrylamide gel in parallel with the immunoprecipitations and Western blot analyses using the anti-E2F-1 and anti-p62 antibodies, as described above.

Degradation assay

Point mutations within the E2F-1 activation domain were obtained by using the Quickchange™ mutagenesis kit (Stratagene) on the pHKGal4 E2F-1 (amino acids 380–437) template (Hagemeier *et al.*, 1993). Ser403 was mutated into Ala (mutation S→A) or into Asp (mutation S→D). Thr433 was also mutated into Ala (mutation T→A) and into Asp (mutation T→D). Finally, double mutations on Ser403 and Thr433 into Ala or Asp were also generated (mutations S/T→A/A and S/T→D/D, respectively).

A 5 µg aliquot of wild-type or point-mutated pHKGal4-E2F-1 (AD) constructs or pHKGal4 vector alone as a control were transfected into a 145 mm dish of 293T cells as previously described. After 16 h, cells were washed twice in PBS and split into five 100-mm dishes. After a

further 24 h, DMEM containing 25 µg/ml of cycloheximide (Sigma) was added to the cells, determining the time 0, and one dish of cells was scraped every 30 min during 2 h for each transfection. The pellets were washed twice in PBS and frozen in liquid nitrogen. Cells were all lysed at the same time in 150 µl of a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 0.5 mM EGTA and 30 U of RNase free-DNase I (Boehringer Mannheim) and were boiled for 5 min. SDS loading buffer was then added and 25 µl of the extracts were loaded on a 12% SDS-polyacrylamide gel. Expression of the fusion proteins was assessed by Western blot analysis using the polyclonal anti-E2F-1 C20 antibody or the anti-Gal4 antibody.

Transactivations

293T cells were transfected with 1 µg of reporter plasmid pBS 5×Gal4 which consists of five Gal4 DNA-binding sites upstream of the minimal adenovirus major late promoter M2 driving the expression of the luciferase gene (a kind gift from H.Stunnenberg) and with 1 µg of the wild-type and mutated pHKGal4-E2F-1 (AD) constructs in the presence of 0.5 µg of the empty vector pCMV or 0.5 µg of pCMV-RB (Hagemeier *et al.*, 1993). The transactivation activity was analysed by luciferase assay according to the manufacturer's instructions (Promega).

Acknowledgements

We thank J.-M.Egly for the generous gifts of purified CAK, purified HAP-TFIIH and anti-p62 monoclonal antibodies. We thank T.Mäkelä for providing pcDNA3 cdk7 dominant-negative expression vector, J.Pines for supplying the His-cdk2 protein, W.S.Dynan for the GST-C-terminal domain of RNA polymerase II plasmid, S.Roberts for the recombinant subunits of TFIIIF, rap74 and rap30, and H.Stunnenberg for the reporter plasmid pBS 5×Gal4 M2-luciferase. M.G.Poupinot, J.Pines and J.Rous are thanked for critically reading the manuscript and for helpful discussions. This work was supported by grants from the Medical Research Council (DR24) and from the Cancer Research Campaign (DR11). L.V. was supported by a grant from the Medical Research Council (DR24).

References

- Adamczewski,J.P., Rossignol,M., Tassan,J.P., Nigg,E.A., Moncollin,V. and Egly,J.M. (1996) MAT-1, cdk7 and cyclin H form a kinase complex which is UV light-sensitive upon association with TFIIH. *EMBO J.*, **15**, 1877–1889.
- Akoulitchchev,S. and Reinberg,D. (1998) The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of Cdk7. *Genes Dev.*, **12**, 3541–3550.
- Amati,B., Alevizopoulos,K. and Vlach,J. (1998) MYC and the cell cycle. *Frontiers Biosci.*, **3**, 250–268.
- Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J. and Kouzarides,T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597–601.
- Brown,A.J., Jones,T. and Shuttleworth,J. (1994) Expression and activity of p40^{Mo15}, the catalytic subunit of cdk-activating kinase, during *Xenopus* oogenesis and embryogenesis. *Mol. Biol. Cell.*, **5**, 921–932.
- Campanero,M.R. and Flemington,E.K. (1997) Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc. Natl Acad. Sci. USA*, **94**, 2221–2226.
- Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M. and Nevins,J.R. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell*, **65**, 1053–1061.
- Clurman,B.E., Sheaff,R.J., Thress,K., Groudine,M. and Roberts,J.M. (1996) Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.*, **10**, 1979–1990.
- Dalton,S. (1992) Cell cycle regulation of the human cdc2 gene. *EMBO J.*, **11**, 1797–1804.
- Diehl,J.A., Zindy,F. and Sherr,C.J. (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.*, **11**, 957–972.
- Dynlacht,B.D., Flores,O., Lees,J.A. and Harlow,E. (1994) Differential regulation of E2F trans-activation by cyclin-cdk2 complexes. *Genes Dev.*, **8**, 1772–1786.
- Dyson,N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.*, **12**, 2245–2262.
- Ellis,M., Chew,Y.P., Fallis,L., Freddersdorf,S., Boshoff,C., Weiss,R.A., Lu,X. and Mittnacht,S. (1999) Degradation of p27^{KIP} cdk inhibitor

- triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. *EMBO J.*, **18**, 644–653.
- Ewen,M.E., Sluss,H.K., Sherr,C.J., Matsushime,H., Kato,J. and Livingston,D.M. (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell*, **73**, 487–497.
- Fagan,R.K., Flint,K.J. and Jones,N. (1994) Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. *Cell*, **78**, 799–811.
- Geng,Y., Eaton,E.N., Picon,M., Roberts,J.M., Lundberg,A.S., Gifford,C., Sardet,C. and Weinberg,R.A. (1996) Regulation of cyclin E transcription of E2Fs and retinoblastoma protein. *Oncogene*, **12**, 1173–1180.
- Gerard,M., Fisher,L., Moncollin,V., Chipoulet,J.M., Chambon,P. and Egly,J.M. (1991) Purification and interaction properties of the human RNA polymerase B (II) general transcription factor BTF2. *J. Biol. Chem.*, **266**, 20940–20945.
- Hagemeier,C., Cook,A. and Kouzarides,T. (1993) The retinoblastoma protein binds E2F residues required for activation *in vivo* and TBP binding *in vitro*. *Nucleic Acids Res.*, **21**, 4998–5004.
- Hateboer,G., Kerkhoven,R.M., Shvarts,A., Bernards,R. and Beijersbergen,R.L. (1996) Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev.*, **10**, 2960–2970.
- Helin,K. (1998) Regulation of cell proliferation by the E2F transcription factors. *Curr. Opin. Genet. Dev.*, **8**, 28–35.
- Hiebert,S.W., Lipp,M. and Nevins,J.R. (1989) E1A-dependent transactivation of the human MYC promoter is mediated by the E2F factor. *Proc. Natl Acad. Sci. USA*, **86**, 3594–3598.
- Hiebert,S.W., Blake,M., Azizkhan,J. and Nevins,J.R. (1991) Role of E2F transcription factor in E1A-mediated transactivation of cellular genes. *J. Virol.*, **65**, 3547–3552.
- Hiebert,S.W., Chellapan,S.P., Horowitz,J.M. and Nevins,J.R. (1992) The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev.*, **6**, 177–185.
- Hofmann,F. and Livingston,D. (1996) Differential effects of cdk2 and cdk3 on the control of pRB and E2F function during G₁ exit. *Genes Dev.*, **10**, 851–861.
- Hofmann,F., Martelli,F., Livingston,D.M. and Wang,Z. (1996) The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. *Genes Dev.*, **10**, 2949–2959.
- Hsiao,K., McMahon,S.L. and Farnham,P.J. (1994) Multiple DNA elements are required for the growth regulation of the mouse E2F-1 promoter. *Genes Dev.*, **8**, 1526–1537.
- Johnson,D.G., Schwarz,J.K., Cress,W.D. and Nevins,J.R. (1993) Expression of transcription factor E2F-1 induces quiescent cells to enter S phase. *Nature*, **365**, 349–352.
- Johnson,D.G., Ohtani,K. and Nevins,J.R. (1994) Autoregulatory control of E2F-1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev.*, **8**, 1514–1525.
- Kaelin,W.G.J. *et al.* (1992) Expression cloning of a cDNA encoding a retinoblastoma binding protein with E2F-like properties. *Cell*, **70**, 351–354.
- Kato,J., Matsushime,H., Hiebert,S.W., Ewen,M.E. and Sherr,C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRB) and pRB phosphorylation by the cyclin D-dependent kinase cdk4. *Genes Dev.*, **7**, 331–342.
- Kitagawa,M., Higashi,H., Suzuki-Takahashi,I., Segawa,K., Hanks,S.K., Taya,Y., Nishimura,S. and Okuyama,A. (1995) Phosphorylation of E2F-1 by cyclin A/cdk2. *Oncogene*, **10**, 229–236.
- Krek,W. (1998) Proteolysis and the G₁-S transition: the SCF connection. *Curr. Opin. Genet. Dev.*, **8**, 36–42.
- Krek,W., Ewen,M.E., Shirodkar,S., Arany,Z., Kaelin,W.G.J. and Livingston,D.M. (1994) Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. *Cell*, **78**, 161–172.
- Krude,T., Jackman,M., Pines,J. and Laskey,R.A. (1997) Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell*, **88**, 109–119.
- Lam,E.W. and Watson,R.J. (1993) An E2F binding site mediates cell cycle regulated repression of mouse B-myb transcription. *EMBO J.*, **12**, 2705–2713.
- Lundberg,A.S. and Weinberg,R.A. (1998) Functional inactivation of the retinoblastoma protein requires sequential modification of at least two distinct cyclin-cdk complexes. *Mol. Cell. Biol.*, **18**, 753–761.
- Luo,R.X., Postigo,A.A. and Dean,D.C. (1998) RB interacts with histone deacetylase to repress transcription. *Cell*, **92**, 463–473.
- Lutterbach,B. and Hann,S.R. (1994) Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. *Mol. Cell. Biol.*, **14**, 5510–5522.
- Magnaghi-Jaulin,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troelen,F., Trouche,D. and Harel-Bellan,A. (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature*, **391**, 601–605.
- Mäkelä,T.P., Parvin,J.D., Kim,J., Huber,L.J., Sharp,P.A. and Weinberg,R.A. (1995) A kinase-deficient transcription factor TFIIF is functional in basal and activated transcription. *Proc. Natl Acad. Sci. USA*, **92**, 5174–5178.
- Mann,D.J., Child,E.S., Swanton,C., Laman,H. and Jones,N. (1999) Modulation of p27^{KIP1} levels by cyclin encoded by Kaposi's sarcoma-associated herpesvirus. *EMBO J.*, **18**, 654–663.
- Martin,K., Trouche,D., Hagemeier,C., Sørensen,T.S., La Thangue,N.B. and Kouzarides,T. (1995) Stimulation of E2F-1/DP-1 transcriptional activity by MDM2 oncoprotein. *Nature*, **375**, 691–694.
- Matsuoka,M., Kato,J.Y., Fisher,R.P., Morgan,D.O. and Sherr,C.S. (1994) Activation of cyclin-dependent kinase 4 (cdk4) by mouse MO15-associated kinase. *Mol. Cell. Biol.*, **14**, 7265–7275.
- Meijer,L. (1995) Chemical inhibitors of cyclin-dependent kinases. *Prog. Cell Cycle Res.*, **1**, 351–363.
- Mittnacht,S. (1998) Control of pRB phosphorylation. *Curr. Opin. Genet. Dev.*, **8**, 21–27.
- Mudryj,M.S., Hiebert,S.W. and Nevins,J.R. (1990) A role for the adenovirus inducible E2F transcription factor in a proliferation-dependent signal transduction pathway. *EMBO J.*, **9**, 2179–2184.
- Nguyen,H., Gitig,D.M. and Koff,A. (1999) Cell-free degradation of p27^{KIP1}, a G₁ cyclin-dependent kinase inhibitor, is dependent on cdk2 activity and the proteasome. *Mol. Cell. Biol.*, **19**, 1190–1201.
- O'Connor,D.J., Lam,E.W., Griffin,S., Zhong,S., Leighton,L.C., Burbidge,S.A. and Lu,X. (1995) Physical and functional interaction between DP-1 and p53 and cell cycle co-operating transcription factors, E2F-1 and DP-1. *EMBO J.*, **14**, 6184–6192.
- Ohtani,K., DeGregori,J. and Nevins,J.R. (1995) Regulation of the cyclin E gene by transcription factor E2F. *Proc. Natl Acad. Sci. USA*, **92**, 12146–12150.
- Patton,E.E., Willems,A.R. and Tyers,M. (1998) Combinatorial control in ubiquitin-dependent proteolysis: don't skip the F-box hypothesis. *Trends Genet.*, **14**, 236–243.
- Pearson,A. and Greenblatt,J. (1997) Modular organization of the E2F-1 activation domain and its interaction with general transcription factors TBP and TFIIF. *Oncogene*, **15**, 2643–2658.
- Peeper,D.S., Keblusek,P., Helin,K., Toebes,M., van der Eb,A.J. and Zantema,A. (1995) Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding *in vitro*. *Oncogene*, **10**, 39–48.
- Peterson,S.R., Dvir,A., Anderson,C.W. and Dynan,W.S. (1992) DNA binding provides a signal for phosphorylation of the RNA polymerase II hexapeptide repeats. *Genes Dev.*, **6**, 426–438.
- Poon,R.Y.C., Yamashita,K., Adamczewski,J.P., Hunt,T. and Shuttleworth,J. (1993) The cdc2-related protein p40^{Mol15} is the catalytic subunit of a protein kinase that can activate p33^{cdk2} and p34^{cdc2}. *EMBO J.*, **12**, 3123–3132.
- Pulverer,B.J., Fisher,C., Vousden,K., Littlewood,T., Evan,G. and Woodgett,J.R. (1994) Site-specific modulation of c-Myc cotransformation by residues phosphorylated *in vivo*. *Oncogene*, **9**, 59–70.
- Rao,P.N. and Johnson,R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature*, **225**, 159–164.
- Rossignol,M., Kolb-Cheynel,I. and Egly,J.M. (1997) Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIF. *EMBO J.*, **16**, 1628–1637.
- Roy,R., Adamczewski,J.P., Seroz,T., Vermuelen,W., Tassan,J.P., Schaeffer,L., Nigg,E.A., Hoeijmakers,J.H.J. and Egly,J.M. (1994) The MO15 cell cycle kinase is associated with the TFIIF transcription-DNA repair factor. *Cell*, **79**, 1093–1101.
- Salghetti,S.E., Kim,S.Y. and Tansey,W.P. (1999) Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *EMBO J.*, **18**, 717–726.
- Schulze,A., Zerfass,K., Spitkovsky,D., Middendorp,S., Berges,J., Helin,K., Jansen-Durr,P. and Henglein,B. (1995) Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. *Proc. Natl Acad. Sci. USA*, **92**, 11264–11268.
- Serizawa,H., Conaway,J.W. and Conaway,R.C. (1993) Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. *Nature*, **363**, 371–374.
- Shan,B., Zhu,X., Chen,P.L., Durfee,T., Yang,Y., Sharp,D. and Lee,W.-H. (1992) Molecular cloning of cellular genes encoding retinoblastoma-

- associated proteins: identification of a gene with properties of the transcription factor E2F. *Mol. Cell. Biol.*, **12**, 5620–5631.
- Shiekhattar,R., Mermelstein,F., Fisher,R.P., Drapkin,R., Dynlacht,B., Wessling,H.C., Morgan,D.O. and Reinberg,D. (1995) Cdk-activating kinase (CAK) complex is a component of human transcription factor IIH. *Nature*, **374**, 283–287.
- Slansky,J.E., Li,Y., Kaelin,W.G.J. and Farnham,P.J. (1993) A protein synthesis-dependent increase in E2F-1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. *Mol. Cell. Biol.*, **13**, 1610–1618.
- Svejstrup,J.Q., Vichi,P. and Egly,J.M. (1996) The multiple roles of transcription/repair factor TFIIH. *Trends Biochem. Sci.*, **20**, 402–405.
- Tassan,J.P., Schultz,S.J., Bartek,J. and Nigg,E.A. (1994) Cell cycle analysis of the activity, subcellular localization and subunit composition of human CAK (Cdk-activating kinase). *J. Cell. Biol.*, **127**, 467–478.
- Trouche,D. and Kouzarides,T. (1996) E2F-1 and E1A 12S have a homologous activation domain regulated by RB and CBP. *Proc. Natl Acad. Sci. USA*, **93**, 1439–1442.
- Won,K.A. and Reed,S.I. (1996) Activation of cyclin E/cdk2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *EMBO J.*, **15**, 4182–4193.
- Xu,M.K., Sheppard,K.A., Peng,C.Y., Yee,A.S. and Piwnica-Worms,H. (1994) Cyclin A/cdk2 binds directly to E2F-1 and inhibits the DNA binding activity of E2F-1/DP-1 by phosphorylation. *Mol. Cell. Biol.*, **14**, 8420–8431.
- Yankulov,K.Y. and Bentley,D.L. (1997) Regulation of cdk7 substrate specificity by MAT-1 and TFIIH. *EMBO J.*, **16**, 1638–1646.
- Yankulov,K.Y., Yamashita,K., Roy,R., Egly,J.-M. and Bentley,D.L. (1995) The transcriptional elongation inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole inhibits transcription factor IIH-associated kinase. *J. Biol. Chem.*, **270**, 23922–23925.

*Received March 16, 1999; revised June 11, 1999;
accepted June 15, 1999*