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

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Introduction

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.

protein RB inhibit E2F-1 transcriptional activity (Hiebert et al., 1992; Hagemeyer 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; Hagemeyer 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 Mittnacht, 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 (Peepere 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 (Dynlacht 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 TFIIH requires three of its subunits, cdks, 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 TFIIH complex (Roy et al., 1994; Shiekhattar et al., 1995; Adamczewski et al., 1996). Free CAK and CAK associated with the core TFIIH complex display different substrate specificity. For example, TFIIH(rap74) and TFII-E(p56) are phosphorylated by TFIIH but not by CAK. Histone H1 and the C-terminal domain of RNA polymerase II (RNA polII CTD) are a better substrate for TFIIH than for CAK, but cdk2 is better phosphorylated by CAK than by TFIIH (Rossignol et al., 1997; Yankulov and Bentley, 1997). However, unlike many other cdk5, cdk7, alone or as part of TFIIH, 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 TFIIH-associated kinase and transcription activities are inhibited (Akoulitchev 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 TFIIH, but not CAK, can phosphorylate E2F-1 (AD) and that the major E2F-1 (AD) kinase activity in cell extracts corresponds to TFIIH. 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 cells extracts is reduced during G1 and is inhibited by RB in vitro. Moreover, we showed that the p62 subunit of the TFIIH complex associates specifically with E2F-1 (AD) in S phase in vivo. Finally, we found that mutation of the two TFIIH phosphorylation sites to non-phosphorylatable residues stabilizes E2F-1. These data suggest that E2F-1 is phosphorylated by TFIIH 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 TFIIH but not purified CAK phosphorylates the E2F-1 activation domain in vitro
As TFIIH 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 TFIIH 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 TFIIH 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 TFIIH 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 TFIIH. GST alone was not phosphorylated by any of these kinases. To confirm the specificity of E2F-1 (AD) phosphorylation by TFIIH, 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 TFIIH. No detectable phosphorylation could be observed, suggesting a specificity for E2F-1 (AD) recognition of TFIIH (data not shown).

Purified TFIIH phosphorylates Ser403 and Thr433 of E2F-1 (AD)
In order to identify the sites phosphorylated by TFIIH, 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 TFIIH (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
The cdk7 component of TFIIH binds the RB-binding site of E2F-1

To determine if the cdk7 subunit of TFIIH could possibly be the kinase which phosphorylates 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 TFIIH 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 TFIIH (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-isouquinoline sulfonamide dihydrochloride), another inhibitor of TFIIH 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 cdks in the phosphorylation of the E2F-1 (AD), we used two compounds, roscovitine and olomoucine, which are potent chemical inhibitors of cdks (cdc2, cdk2, cdk5 and, to a lesser extent, cdk6) (Meijer, 1995). No effect on E2F phosphorylation was observed when these compounds
Degradation of E2F-1 by phosphorylation

Fig. 2. Purified TFIIH, 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 TFIIH 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 TFIIH. 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 TFIIH, 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 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 TFIIH 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 were assessed for kinase activity associated with E2F-1 (AD) using pull-down kinase assays. In the presence of 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 TFIIH substrates

Next, we tested whether the E2F-1 (AD)-interacting kinase could phosphorylate substrates previously reported to be phosphorylated efficiently by TFIIH. 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 TFIIF, 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 TFIIF 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.
Degradation of E2F-1 by phosphorylation

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 TFIIH 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 immunoprecipitations throughout the cell cycle were quantitative. When endogenous E2F-1 immunoprecipitates were immuno-blotted for the presence of p62, only extracts from cells synchronized in early S phase showed a significative amount of associated p62 (Figure 6D). These results 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
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 Flemington, 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-
rlation 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 cdks or MAPK phosphorylation sites. We show that this phosphorylation is carried out by the TFIIH 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 TFIIH kinase in vitro, demonstrating that TFIIH 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 TFIIH, 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 TFIIH, binds to E2F-1 at the RB-binding site and displaces RB (Pearson and Greenblatt, 1997). This finding may explain the fact that purified TFIIH, but not purified Cdk7, which lacks the p62 subunit, phosphorylated E2F-1 (AD) in vitro. Such a difference in substrate specificity between TFIIH and Cdk7 has also been described for the general transcription factors TFIIF (rap74 subunit) and TFIIE (p56 subunit), which are phosphorylated by TFIIH but not by Cdk7 (Yankulov and Bentley, 1997). One of the subunits of TFIIH which could play the role of a bridge between E2F-1 (AD) and TFIIH 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 TFIIH 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 TFIIH in E2F-1 (AD) phosphorylation was obtained using an inhibitor of TFIIH 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 TFIIH (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 TFIIH 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 TFIIH) and cdk7 were pulled down with the E2F-1 (AD). Altogether, these results strongly suggest that E2F-1 (AD) is phosphorylated by TFIIH kinase in vitro. Among the cdks, only Cdk7/TFIIH kinase has been shown to be constitutively active during G1, S and G2 phases (Tassan et al., 1994). As purified TFIIH 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 TFIIH 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., 1994; Xu et al., 1994) 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 (Dylnacht 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 in
of RB binding, since RB could repress the transcriptional activity of all the mutants to the same extent (Figure 7B). Because TFIH binds to E2F-1 specifically in S phase in vivo when E2F-1 is degraded rapidly, and because TFIH 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 TFIH 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 p27KIP (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 pol II CTD was expressed in E. coli as described in Gerard et al. (1991) and 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 Phosphorimag. 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 phenylmethylsulfonil fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupetin and 1 μg/ml pepstatin] and phosphatase inhibitors as described above. A 250 μl 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 Phosphorimag.

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 phosphorimpat rap74, rap30 and subunits of TFIIH (gift from S.Roberts), 50 ng of His-cdk2 (gift from J.Pines) and 100 ng of histone H1 (Boehringer Mannheim). A 50 μg 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-2 (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 μM/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 Phosphorimag.

**Western blots and immunodepletion**

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-

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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 (Mäkela 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 10-cm 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 G1 by releasing the thymidine-blocked cells in early S phase into fresh 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 G2), 6 (mid-G2) or 10 h (late G2). 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 (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 rebotted with the monoclonal anti-E2F-1 antibody HK95 (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 pHKGal4E2F-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 pHKGal4E2F-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 transcription. 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 pHKGal4E2F-1 (AD) constructs in the presence of 0.5 μg of the empty vector PCMv or 0.5 μg of PCMv-RB (Hagemeyer et al., 1993). The transactivation activity was analysed by luciferase assay according to the manufacturer’s instructions (Promega).

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