Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner

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Cyclin E is classified as a putative G₁ cyclin on the basis of its cyclic pattern of mRNA expression, with maximal levels being detected near the G₁/S boundary. We report here that cyclin E is found associated with the transcription factor E2F in a temporally regulated fashion. E2F is known to be a critical transcription factor for the expression of some S phase-specific proteins and is thought to be important for a series of others. Antisera specific for cyclin E were raised and used to demonstrate an association between cyclin E and E2F. This cyclin E/E2F complex was seen in a variety of human cell lines from various tissues, but its appearance was detected primarily during the G₁ phase of the cell cycle. The cyclin E/E2F association decreased as cells entered S phase, just as the association of E2F with cyclin A became detectable. We characterized the cyclin E–E2F complex further to show that both the cyclin-dependent kinase-2 (cdk2) and p107 were present. Therefore, the p107/E2F complex is associated with two different cdk2 kinase complexes—one containing cyclin A and the other containing cyclin E—and the appearance of these complexes is temporally regulated during the cell cycle. The presence of cyclin E/E2F complexes in the G₁ phase suggests a role for cyclin E in the control of genes required for the G₁-to-S transition.

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Our understanding of the control of cell division in mammalian cells has stemmed largely from work carried out in other systems, particularly in the analysis of cell cycle control in yeast. Studies both in budding and fission yeast have demonstrated important control points committing cells to enter S phase and mitosis (Hartwell 1973; Nurse 1975). Attempts to identify the proteins regulating these two commitment points have revealed elaborate pathways involving many components. The protein kinase cdc2 (or Cdc28 in *Saccharomyces cerevisiae*) appears to be required at both regulatory points (Nurse and Bissett 1981; Piggott et al. 1982; Reed and Wittenberg 1990). The activity of this kinase is controlled through association with regulatory subunits known as cyclins (Booher et al. 1989; Draetta et al. 1989; Moreno et al. 1989; Richardson et al. 1989; Wittenberg et al. 1990).

Cyclins were first identified in marine invertebrates as proteins with levels that oscillate dramatically through the cell cycle (Evans et al. 1983; Swenson et al. 1986; Standart et al. 1987; Hunt et al. 1992). These proteins have been well conserved through evolution and play a critical role in the regulation of cellular division (Swenson et al. 1986; Minshull et al. 1989; Murray et al. 1989;

Solomon et al. 1990). The G₂/M transition is dependent on the activity of the cdc2 kinase, the activation of which requires association of cdc2 with B-type cyclins (Booher et al. 1989; Draetta et al. 1989; Moreno et al. 1989). Homologs of this kinase/cyclin complex appear to regulate the G₂/M transition in all eukaryotic cells.

In addition to their role in controlling entry into mitosis, cyclins have also been shown to control earlier stages of the cell cycle. In mammalian cells, a cyclin A-associated kinase appears in interphase, probably near the beginning of S phase (Giordano et al. 1989; Pines and Hunter 1990; Pagano et al. 1992b). Microinjection of either antisense oligonucleotides or antibodies against cyclin A causes a block or delay in DNA synthesis (Girard et al. 1991; Pagano et al. 1992b). Other data showing the requirement for cyclin A for entry into mitosis would suggest a later function for cyclin A at the G₂/M boundary (Lehner and O'Farrell 1990; Walker and Maller 1991; Pagano et al. 1992b). In *S. cerevisiae* there are regulatory proteins that clearly control progression through G₁, by modulating the activity of the cdc2 homolog, Cdc28 (Reed 1991). These G₁ cyclins are known as Cln1, Cln2, and Cln3 (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989; Richardson et al. 1989; Wittenberg et al. 1990) and share limited homology with the mitotic cyclins in the most conserved region of the cyclin protein, known as

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the cyclin box, which is the region required to bind to cdc2 (E. Lees and E. Harlow, in prep.; S. Shiff and E. Harlow, in prep.; H. Kobayashi, E. Stewart, and T. Hunt, pers. comm.).

Until recently, mammalian equivalents to these G_1 cyclins had not been identified, and it has therefore been unclear whether cyclins have any role to play in controlling G_1 events in multicellular organisms. Several novel mammalian cyclins—C, D1, and E—have now been isolated by virtue of their ability to rescue loss of CLN function mutations in yeast (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991). Cyclin D1 was also isolated independently as an amplified region of DNA in parathyroid tumors (Motokura et al. 1991) and as a gene that was stimulated by colony stimulating factor treatment of murine macrophages (Matsushime et al. 1991). Because the mitotic cyclin B1 can also rescue the loss-of-function CLN mutation in yeast, this screen alone is clearly not selective for G_1 cyclins. However, these new cyclins appear to be good candidates for functioning in G_1 , given the cyclic pattern of their RNA expression with levels peaking at the G_1/S boundary (Lew et al. 1991; Matsushime et al. 1991; Motokura et al. 1991).

Many issues exist regarding regulation and events that occur in mammalian cells during G_1 and at the G_1/S transition. Of particular importance has been the identification of transcription factors that mediate cell cycle-dependent gene control. Transcription of a number of genes encoding proteins that are required in G_1 and S occurs only transiently during G_1 . Therefore, this time is also an important point for transcriptional control (for review, see Pardee 1989). The transcription factor E2F has been implicated in the regulation of such genes. E2F-binding sites have been identified in the 5'-flanking region of a number of early genes, including *c-myc*, *N-myc*, and the dihydrofolate reductase (DHFR) gene and have been shown to be important for their transcription (Blake and Azizkhan 1989; Hiebert et al. 1989, 1991a; Mudryj et al. 1990). The activity of E2F is thought to be tightly regulated by association with other proteins, including the protein product of the retinoblastoma gene pRB and a structurally related protein, p107 (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991; Raychaudhuri et al. 1991; Cao et al. 1992; Shirodkar et al. 1992). Several studies have implicated pRB as a repressor of transcription (Laiho et al. 1990; Pietenpol et al. 1990), and it is speculated that pRB association with E2F prevents transcriptional activity (Bandara and La Thangue 1991; Chellappan et al. 1991; Hiebert et al. 1991b; Raychaudhuri et al. 1991). The existence of a complex between p107, cyclin A, cyclin-dependent kinase-2 (cdk2), and E2F during S phase suggests a direct involvement of components of the cell cycle machinery in the regulation of E2F (Bandara et al. 1991; Mudryj et al. 1991; Cao et al. 1992; Pagano et al. 1992a; Shirodkar et al. 1992). The timing of the appearance of this cyclin A complex suggests that other regulatory events must control the onset of E2F transcriptional activity in G_1 .

The newly isolated cyclins are good candidates for

such regulators, given the timing of their expression during G_1 and the precedence for cyclin association with E2F based on observations made for cyclin A. We set out to investigate whether any of the newly isolated cyclins were associated with E2F, particularly during the G_1 phase of the cell cycle. Using antibodies raised against human cyclin E, we have been able to describe a unique activity for cyclin E, which suggests its involvement in the regulation of the transcription factor E2F in a temporally distinct manner from cyclin A.

Results

Antisera specific for human cyclin E

To study cyclin E and its associated proteins, we raised antibodies specific for cyclin E. To prepare an appropriate immunogen, human cyclin E was purified from two sources: (1) Full-length cyclin E was synthesized in bacteria from a T7 expression vector [(Studier et al. 1990); the construction of the cyclin E vector and the methods for purification of the protein are described elsewhere (Dulic et al. 1992)], or (2) a fusion protein between cyclin E and glutathione *S*-transferase (GST) was purified as described previously (Smith and Johnson 1988). These proteins were used as immunogens in BALB/c mice. Initially, antisera were tested by immunoprecipitation of cyclin E polypeptides synthesized by *in vitro* translation of cRNA prepared from the human cyclin E cDNA. Cyclin E antisera were not able to recognize the *in vitro* translation products of cyclins A, B1, B2, C, and D1 (data not shown).

After a strong immune response developed, the antibodies were tested for specificity to cyclin E by immunoblots (Fig. 1A) and immunoprecipitations of metabolically labeled cells (Fig. 1B). For immunoblots, whole-cell extracts were prepared from asynchronously growing ML-1 cells (a myeloid leukemia cell line) (lanes 1) and from ML-1 cells arrested at the G_1/S boundary by a double thymidine block (lanes 2) (Rao and Johnson 1970). Figure 1A shows that the mouse polyclonal antiserum raised against cyclin E recognized a single polypeptide species migrating with a relative molecular mass of ~51 kD. A band of this same size was also detected by use of an anti-cyclin E rabbit antiserum. The *in vitro* translation product of cyclin E migrated with a similar mobility (data not shown). The levels of this protein were elevated significantly in double thymidine-blocked cells (lanes 2), as has been observed in independent experiments by Dulic and colleagues (1992). The mouse antiserum failed to recognize any other proteins, indicating that there is no strong cross-reactivity with other denatured proteins, particularly with other cyclins. A duplicate blot probed with a cyclin A monoclonal antibody, BF683 (Faha et al. 1992a), showed similarly elevated levels of cyclin A in the drug-treated cells. This also served as a good control for the specificity of the cyclin E antibodies, as they failed to recognize cyclin A at this easily detectable level.

To test the ability of the antisera to recognize native cyclin E, we performed immunoprecipitations from de-

tergent lysates of metabolically labeled cells (Fig. 1B). Cyclin E antisera specifically precipitated a broad band of polypeptides migrating with apparent molecular masses of 48–51 kD (lanes 2,3). Given the low abundance of cyclin E and its heterogeneous nature, a long exposure of the gel to film was required to visualize the putative cyclin E band clearly. At this exposure a number of other polypeptides that were precipitated with these antisera were also intensified. We therefore performed immunoprecipitations in parallel on denatured lysates (lanes 7–9) to discriminate between associated proteins and polypeptides with cross-reactive epitopes. Lane 7 shows that the cyclin E antiserum recognized only two polypeptides specifically: the broad 48- to 51-kD species and a faster migrating 46-kD species. To confirm the identity of the slower migrating protein as cyclin E, we performed V8 protease partial proteolytic mapping on the protein excised from a SDS-polyacrylamide gel and compared the pattern of digestion with similarly treated cyclin E synthesized in vitro. Both gave identical patterns (Fig. 1C),

confirming that this broad band was cyclin E. The identity of the additional 46-kD polypeptide seen in lane 7 is not known but may be a degradation product of cyclin E resulting during the denaturation process. A protein migrating with similar but somewhat slower mobility to cyclin E was seen in anti-cyclin A immunoprecipitates (lane 5); this proved to be distinct from cyclin E by proteolytic mapping (data not shown). The appearance of a broad band suggested that cyclin E was post-translationally modified to produce variable migrating species. Cyclin E immunoprecipitates performed on cells labeled with orthophosphate (Fig. 1D, lane 2) indicated that at least one of these forms was phosphorylated.

The cyclin E antibodies also coprecipitated a polypeptide of ~33 kD. This band migrated with similar mobility to cdk2, as seen in an anti-cyclin A immunoprecipitation (Fig. 1B, lane 5) and in an anti-E1A immunoprecipitation (Fig. 1D, lane 1); immunochemical and proteolytic comparisons confirmed this identification (L.-H. Tsai, E. Harlow, and E. Lees, in prep.). Thus, cyclin

E interacted with at least cdk2, although we cannot rule out the possibility that cyclin E associates with other, uncharacterized catalytic subunits. In addition, a protein of ~107 kD coprecipitated with the anti-cyclin E antisera (Fig. 1B, lanes 2,3; Fig. 1D, lane 2), which comigrated with p107 observed in anti-cyclin A immunoprecipitations (Fig. 1B, lane 5) and in anti-E1A immunoprecipitations from 293 cells (Fig. 1D, lane 1). The identification of this protein as p107 is discussed in a later section.

E2F coprecipitates with cyclin E

Both cyclins A and E are now known to bind to cdk2 (Tsai et al. 1991; Dulic et al. 1992; Faha et al. 1992b). The cyclin A/cdk2 complex has been described previously, and two assays have been described that characterize its activities. These include the temporal regulation of its kinase activity (Giordano et al. 1989; Pines and Hunter 1990; Pagano et al. 1992b; Rosenblatt et al. 1992) and the association with the transcription factor E2F (Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992). Both of these activities become detectable around the initiation of DNA synthesis. Recent work has shown that the cyclin E/cdk2 complex has a cell cycle-associated kinase activity that is activated in mid-to-late G₁, well before the initiation of DNA synthesis or the activation of cyclin A/cdk2 kinase activity and will be described elsewhere (Dulic et al. 1992; L.-H. Tsai, E. Harlow, and E. Lees, in prep.). In this study we were interested in investigating the involvement of other cyclins in the regulation of E2F activity.

To detect an association between cyclin E and E2F, cyclin E was immunoprecipitated from several different cell lines, ML-1, CEM (T lymphoblast), HL60 (myeloid leukemia), and MANCA (Burkitt's lymphoma), and immunocomplexes were tested for the presence of E2F. Figure 2A shows that the cyclin E antiserum coprecipitated E2F activity in the cell lines tested, indicating that there was an association between cyclin E and E2F. Similar experiments with anti-pRB [XZ77 (Hu et al. 1991); lane 1] and anti-cyclin A [C160 (Giordano et al. 1989); lane 2] were included as positive controls for the presence of E2F. To rule out possible cross-reaction of our anti-cyclin E reagents with cyclin A, cell extracts were pre-cleared of cyclin A using a monoclonal antibody against cyclin A (C160). As a negative control, lysates were pre-cleared with PAb416 (Harlow et al. 1981), a monoclonal antibody against SV40 T antigen. In the absence of cyclin A we detected similar levels of the E2F activity with the cyclin E antiserum as shown in Figure 2B (cf. lanes 3 and 6). An association between cyclin E and E2F was supported further by the observation that a cyclin E GST fusion protein could bind E2F from cell lysates (data not shown).

The association of cyclin E with E2F is regulated in a cell cycle-dependent manner

We then studied the profile of the association of cyclin E with E2F through the cell cycle. Because it has been shown that cyclin E mRNA expression and associated kinase activity are cell cycle regulated with maximal

Figure 2. Association of cyclin E with transcription factor E2F in dividing cells. (A) Cell extracts from a number of different cell lines were immunoprecipitated with the indicated antibodies. Monoclonal antibodies against pRB (XZ77; lane 1), cyclin A (C160; lane 2), and SV40 large T antigen (PAb416; lane 3) were included as controls. Immune complexes were treated with DOC, and the eluate was assayed for E2F activity by a gel retardation assay. A whole-cell extract from 293 cells served as a marker for E2F mobility. (B) Whole-cell extracts from ML-1 cells were pre-cleared of cyclin A using an anti-cyclin A antibody (C160) cross-linked to beads. PAb416 cross-linked beads were used as a negative control. Immunoprecipitations were performed using antibodies as indicated. Immune complexes were disrupted with DOC and assayed for E2F activity as before.

expression and activity at the G₁/S boundary (Lew et al. 1991; Dulic et al. 1992; Tsai et al. 1992), we were interested to learn whether the association of cyclin E and E2F was regulated in a similar fashion. To obtain cells that were enriched for a particular phase of the cell cycle, we tried two methods: (1) treatment of cells with drugs known to arrest at specific stages of the cell cycle, and (2) centrifugal elutriation to separate cells on the basis of cell size. Extracts were prepared from these cells, and cyclin E and cyclin A immunoprecipitations were tested for the presence of E2F as before. Figure 3A shows that

cyclin E is associated with E2F in cells blocked in both late G₁ by mimosine, a plant amino acid analog (Lalande 1990), and in S phase by hydroxyurea. In contrast, the levels of cyclin A association were highest in cells arrested in M phase by the microtubule inhibitory drug nocodazole (lane 12) and were markedly reduced in the earlier blocks. These experiments suggested that the timing of cyclin E association with E2F was different from that observed with cyclin A, occurring earlier in the cell cycle as indicated from elevated levels in G₁-arrested cells.

Figure 3. Cell cycle analysis of E2F/cyclin complexes in drug-arrested ML-1 cells (A) and elutriated HL60 cells (B). (A) Whole-cell extracts were prepared from ML-1 cells, treated with drugs, where indicated. Immunoprecipitations were performed using mouse anti-cyclin E antiserum (anti-E), normal mouse serum (NMS), and a cyclin A monoclonal antibody (C160; anti-A). Immune complexes were disrupted with DOC and assayed for E2F activity. A whole-cell extract from 293 cells served as a marker for E2F mobility. The DNA content of cells from each treatment was determined by FACS analysis to give a measure of the relative percentages of G₁, S, G₂ cells in each sample (bottom). (B) HL60 cells were elutriated, and whole-cell extracts were prepared from various fractions (2–9). Immunoprecipitations were performed using antibodies as indicated and analyzed as described in A.

Previously, we have observed that some drugs, thymidine for example, caused artificially high levels of cyclins to accumulate in the treated cells. Therefore, we used a second method of synchrony, centrifugal elutriation, to confirm these observations as shown in Figure 3B. Several fractions were collected, and extracts were prepared, and subsequently tested for the presence of E2F as before. In earlier fractions containing 85% G₁ cells (fraction 2), only cyclin E was found to be associated with E2F. The levels of cyclin E/E2F complexes decreased in later fractions. In contrast, E2F in cyclin A immunoprecipitations only appeared as extracts became enriched for S-phase cells, being first detectable in fractions containing 85% S-phase cells (fraction 4). Levels of cyclin A-associated E2F activity continued to increase for a further two fractions and then disappeared by fraction 7 (76% G₂ cells). We were surprised to see the reappearance of the cyclin A-associated E2F in the last fraction (9). This pattern has been repeated in two other elutriations (data not shown).

Cyclin E associates with p107 and cdk2 in vivo

Association of E2F with both the product of the retinoblastoma gene product (pRB) and a structurally related protein, p107 (Ewen et al. 1991), have been described previously (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991; Raychaudhuri et al. 1991; Cao et al. 1992; Shirodkar et al. 1992). We were therefore interested to learn whether either of these two proteins was present in the cyclin E/E2F complex. As mentioned earlier immunoprecipitations of cyclin E contained a 107-kD protein that comigrated with p107 coprecipitated with anti-cyclin A antibodies (Fig. 1B; cf. lanes 2 and 3 with lane 5) and with the anti-E1A monoclonal antibody M73 (Fig. 1D) (Harlow et al. 1985). Proteolytic mapping of these 107-kD species showed that they were indistinguishable, thus confirming the identity of the cyclin E-associated protein as p107 (Fig. 4A). We have also been able to detect cyclin E in anti-p107 immunoprecipitations (N. Dyson and E. Lees, unpubl.) providing further evidence for the complex, as detection is possible using reagents toward either component. Interestingly, p107 also appears to be a substrate for a cyclin E-associated kinase (Fig. 4B), showing that p107 could be phosphorylated in an in vitro kinase assay performed on a cyclin E immunoprecipitation. Immunoprecipitates of cyclin A (BF683) and E1A (M73) were included as controls because both are known to contain kinase activity that phosphorylates p107 (Herrmann et al. 1991; Faha et al. 1992a).

Using gel retardation assays, we were able to show the presence of p107 in the cyclin E/E2F complex as shown in Figure 5B. Visualization of the cyclin E/E2F complex by gel retardation proved problematic. It was first necessary to use G₁-enriched fractions from an HL60 elutriation to observe cyclin E complexes. In addition, the anti-cyclin E antiserum had to be diluted to obtain a supershifted complex that entered the gel rather than aggregating in the wells. Under these conditions we were

Figure 4. Cyclin E is associated with p107 in vivo. (A) V8 partial proteolytic mapping of p107. [³⁵S]Methionine-labeled 293 cells were immunoprecipitated with an E1A monoclonal antibody (M73). The p107 polypeptide coprecipitating with E1A was excised together with a comigrating band observed in cyclin E immunoprecipitations from metabolically labeled ML-1 cells. V8 partial proteolysis of the two proteins was performed, and the digested polypeptides were resolved on a 15% SDS-polyacrylamide gel. Three dilutions of the protease were used as indicated. (B) In vitro kinase assays performed on immunoprecipitations, using either 293 cells or ML-1 cells with antibodies, as indicated.

able to detect a complex that was supershifted with anti-cyclin E antisera (Fig. 5A, lanes 3,5) and that migrated slightly slower than the cyclin A-supershifted complex (Fig. 5A, lanes 6,8). The anti-cyclin E antisera could be specifically blocked with the cyclin E GST fusion protein but not by cyclin A GST fusion protein. We were surprised that neither the p107 nor the pRB-containing complexes were depleted by this supershift with cyclin E antibodies. However, we were able to show the presence of p107 in the cyclin E/E2F complex by virtue of the ability of an anti-p107 polyclonal antibody to further retard the anti-cyclin E-supershifted complex (Fig. 5B, lanes 4,5). The cyclin E-containing complexes were unaffected by the addition of control serum or tissue culture supernatant. The inability to appreciably deplete the p107 complex itself, with anti-cyclin E antisera, suggested that we were only detecting a very small fraction of the p107/E2F complexes associated with cyclin E, using this type of assay. We believe that this is a result of the failure of the anti-cyclin E antisera to perform well in gel retardations, with the antisera only being able to shift a small percentage of total cyclin E complexes. The comparatively easy detection of cyclin E association with E2F by immunoprecipitation suggested that the levels of cyclin E/E2F complexes were significant. We are currently generating monoclonal antibodies against cyclin E that will perform better in gel retardation assays. With such reagents it should be possible to obtain a more reliable

Figure 5. Detection of p107 and cdk2 in the cyclin E/E2F complex. Shown is a gel shift analysis of E2F complexes in G_1 -enriched elutriation fraction of HL60 cells. Gel shift reactions were incubated with the indicated antibodies. A 1 : 5 dilution of the cyclin E antiserum was used; 1 μ l of antibody was used in all other incubations. (A) Antibodies were blocked by preincubation with GST fusion proteins as indicated. (B,C) For double antibody reactions, one was added 5 min before the addition of the other in the order stated. The complexes were separated from unbound probe on a 4% polyacrylamide gel. Free probe was run off the bottom of the gel to help resolve the retarded complexes.

estimate of relative amounts of cyclin E/E2F complexes as compared with other G_1 material. The cyclin E-containing complex was not completely shifted with the p107 antiserum (Fig. 5B, lanes 4,5), suggesting that cyclin E may be associated with other E2F complexes.

In similar experiments we investigated the possible presence of a kinase subunit in the cyclin E/E2F complex by looking at the ability of several anti-cdk reagents to alter the cyclin E supershift. Using cdk2-specific antisera (Tsai et al. 1991) we were able to abolish the cyclin E supershift as shown in Figure 5C (lane 5), whereas other antibodies recognizing other cdk-related enzymes had no effect (data not shown). We have additionally been able to show that immunoprecipitations of cyclin E contain cdk2, as shown in Figure 1B. This association has been characterized and will be described elsewhere (Dulic et al. 1992; L.-H. Tsai, E. Harlow, and E. Lees, in prep.). From these data we conclude that cyclin E/E2F complexes contain both p107 and cdk2.

We have performed a number of experiments to investigate the presence of pRB in these cyclin E complexes. We have tested several anti-pRB antibodies for their ability to alter the mobility of the cyclin E complex in a gel

retardation assay, but none had a detectable effect (data not shown). In addition, cyclin E immunoprecipitations from two cell lines, WERI-1 and SAOS-2, which carry defective alleles of the RB-1 gene and therefore cannot produce functional pRB protein, contained substantial levels of E2F activity suggesting that the cyclin E/E2F association is not dependent on the presence of pRB (data not shown). Immunoprecipitations and Western blot analysis, with the reagents available, also failed to identify an association between cyclin E and pRb. We cannot rule out the possibility that cyclin E/Rb/E2F complexes might exist in cell lines carrying wild-type Rb protein; however, if such complexes exist, we have been unable to detect them.

p107 association with E2F through the cell cycle

The presence of cyclin E/p107/E2F complexes in G_1 was surprising given previous models of p107/E2F as an S-phase complex (Cao et al. 1992; Shirodkar et al. 1992). We were therefore keen to learn how the levels of p107 association with E2F changed as a function of cell cycle progression, in comparison to the fluctuations that we

had observed with cyclin A and cyclin E. Immunoprecipitations were performed on elutriated HL60 cells by use of either a polyclonal antiserum against p107 (Cao et al. 1992) or anti-cyclin E and anti-cyclin A reagents as before. Figure 6 shows that p107 was found associated with E2F throughout the cell cycle, albeit at varying levels. Levels were high in the earliest fractions reminiscent of cyclin E, peaking in S phase where levels of cyclin E/E2F were still detectable but where cyclin A/E2F was most abundant, and decreased through G₂ with an increase in the last fraction as is seen with cyclin A. Thus, comparing the profile of p107 to cyclin E and cyclin A, it appears that p107 is a composite of the pattern seen with the two cyclins.

Discussion

Cyclin E association with E2F during G₁

E2F is a critical transcription factor that is needed for the expression of at least one protein (DHFR) essential for DNA replication (Blake and Azizkhan 1989). The appearance of E2F-binding sites in the promoters of other genes

that encode proteins needed for DNA synthesis suggests that the requirement for E2F activation may be a characteristic of many such genes. Consequently, the regulation of E2F activity may help to control the timing of S phase by providing temporal transcriptional regulation. How this regulation might occur is still poorly understood; however, the physical association with cyclin/kinase complexes provides a possible mechanism. This type of control was first suggested by the demonstration of a cyclin A/cdk2/p107/E2F complex during S phase (Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Pagano et al. 1992a; Shirodkar et al. 1992). This work raised the possibility that other cyclin/kinase complexes might function in the regulation of E2F during other stages of the cell cycle. Cyclin E was a good candidate for performing such a role in G₁, given its pattern of expression (Lew et al. 1991) and kinase activity (Dulic et al. 1992; L.-H. Tsai, E. Harlow, and E. Lees, in prep.), both peaking at the G₁/S boundary.

Using cyclin E-specific reagents, we have shown that cyclin E associates with the transcription factor E2F during the G₁ phase of the cell cycle, suggesting a functional role for this cyclin before DNA synthesis. The timing of the appearance of the cyclin E/E2F complex is in marked contrast to the later appearance of the cyclin A/E2F complex (Mudryj et al. 1991; Pagano et al. 1992a; Shirodkar et al. 1992). Given this difference, it was therefore a surprise to learn that the other components of the two cyclin/E2F complexes were the same, namely p107 and cdk2. It is thus apparent that the temporal appearance of the two complexes must be at least one of the keys in determining their potential roles.

The difference in timing of cyclin A and cyclin E association with E2F correlates well with the appearance of the respective proteins through the cell cycle and, interestingly, with the appearance of associated histone H1 kinase activity (Dulic et al. 1992; L.-H. Tsai, E. Harlow, and E. Lees, in prep.). It is therefore attractive to speculate that these kinases are playing an active role in the regulation of E2F activity by phosphorylation of components of the complexes such as pRB, p107, or even E2F itself. p107 appears to be a substrate for both cyclin A and cyclin E, at least in vitro.

New models for E2F regulation

The first models for the roles of pRB and p107 in E2F regulation predicted distinct functions for pRB and p107, during G₁ and S phase, respectively, both acting in the regulation of a single E2F activity. Clearly, our experiments as well as those of others (e.g., Shirodkar et al. 1992) now imply a more complicated scenario. Our observations define two complexes of p107/E2F that are found at different points in the cell cycle. At least one other complex containing pRB is also present, but other complexes may exist as well. A possible example of another is the G₁ complex, tentatively designated as X, described by Shirodkar and colleagues (1992). The demonstration of multiple E2F complexes raises interesting

Figure 6. Cell cycle analysis of p107 complexes in HL60 cells. Whole-cell extracts were prepared from elutriation fractions of HL60 cells. Immunoprecipitations were performed using a mouse anti-cyclin E antiserum, a mouse anti-p107 antiserum (Cao et al. 1992), and a cyclin A monoclonal antibody (C160). Immune complexes were disrupted with DOC and assayed for E2F activity. The DNA content of cells from each fraction was determined by FACS analysis to give a measure of the relative percentages of G₁, S, and G₂ cells in each fraction (bottom).

questions about their respective roles. Three general models must be considered. First, perhaps these complexes regulate the same set of promoters, and the various complexes are responsible for a complicated series of positive and/or negative regulatory decisions that are temporally controlled. Alternatively, the different complexes may regulate E2F in a similar fashion but may direct E2F to different subsets of genes. Finally, these different complexes may identify a series of E2F-like transcription factors that might target different genes or the same genes at different times. Both of the later two models are attractive given the large number of genes that contain E2F sites, and they suggest an elegant scheme for regulation of a diverse set of genes using a relatively small set of players.

It would seem that the p107/E2F complex will be a particularly good system to begin the further analysis of how these various complexes are regulated. The demonstration of the G₁-phase association with cyclin E/cdk2 and the S-phase association with cyclin A/cdk2 should provide a useful comparison to study these complexes. The next important step will be to investigate the ability of the cyclin E and cyclin A complexes to differentially regulate genes containing E2F sites. The cyclins apparently provide an important timing control dictated by the different phases of the cell cycle.

Materials and methods

Antisera

A full-length cyclin E cDNA was cloned into the expression vector pGEX-2T (Pharmacia). This construct was used to transform *Escherichia coli* XA90 cells. The transformed cells were grown to OD₅₅₀ = 0.6 and induced with 0.1 mM IPTG to produce a fusion protein with GST. The fusion protein was purified as described previously (Smith and Johnson 1988). BALB/c mice received repeated injections of 20–50 µg of purified protein. Preparation of rabbit polyclonal cyclin E antisera is described elsewhere (Dulic et al. 1992). The mouse polyclonal cdk2-specific antiserum was kindly provided by L.-H. Tsai (Tsai et al. 1991), and the polyclonal p107 antibodies were provided by S. Dembski (Cao et al. 1992). Monoclonal antibodies used in these studies have all been described previously: BF683 (Faha et al. 1992a); C160 (Giordano et al. 1989); XZ77 (Hu et al. 1991); PAb419 and PAb416 (Harlow et al. 1981). Covalent cross-linking of antibodies to protein A-Sepharose beads was performed as described previously (Harlow and Lane 1988).

Cell lines

ML-1, CEM, HL60, and WERI-1 cells were grown as suspension cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. SAOS-2 cells and 293 cells were grown as monolayers in DMEM supplemented with 10% calf serum.

Synchronization and centrifugal elutriation

ML-1 cells were synchronized by use of a double thymidine block (Rao and Johnson 1970). Cells were treated with 2 mM thymidine for 18 hr and released into fresh media containing 10% FBS for 10 hr, and 2 mM thymidine was added for an ad-

ditional 18 hr. Cells were harvested by centrifugation (1000 rpm/5 min) and washed once in cold PBS, and the pellets were frozen at –70°C until use. Other G₁ arrests were achieved by treating asynchronously growing ML-1 cells with hydroxyurea at a final concentration of 5 µg/ml for 18 hr or by mimosine at 2 mM for 18 hr. Nocodazole was used at a final concentration of 50 ng/ml for 18 hr to arrest cells in G₂. For fractionation of cells by centrifugal elutriation, HL60 cells were grown in suspension at a density of 1 × 10⁶ cells/ml in 10% heat-inactivated FBS (HIFBS). Cells were split the day before elutriation into 20% HIFBS, to give a 4-liter culture at 5 × 10⁵ cells/ml. Approximately 2 × 10⁹ cells in elutriation buffer (Giordano et al. 1989) were loaded onto a Beckman JE5.0 elutriator rotor spinning at 1750 rpm at a rate of 130 ml/min. The centrifugal speed was then adjusted to 1550 rpm, and fractions were collected as the flow rate was increased by increments of 20 ml/min. Cell pellets were frozen at –70°C. Samples were taken to be analyzed for DNA content after staining with propidium iodide by FACS.

Western blotting

Cells were lysed in SDS-sample buffer, sonicated, and heated to 90°C for 10 min. The equivalent of 1.0 A₂₈₀ was separated on a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to Immobilon membranes (Millipore) at 30 V for 3 hr at 4°C, using standard techniques (Sambrook et al. 1989). The immunoblotting was performed as described (Dulic et al. 1992). Primary antibodies were used at 1 : 2000 dilution for polyclonal antisera and 1 : 4 for monoclonal antibody supernatants. Secondary antibodies were either peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulins (Cappel; used at 1 : 5000 dilution). The detection system used was chemiluminescence (ECL; Amersham).

Immunoprecipitations

Immunoprecipitations were performed on cells lysed with buffer containing 250 mM NaCl, 50 mM HEPES (pH 7.0), and 0.1% NP-40, as described previously (Harlow et al. 1985). Lysates were denatured by the addition of SDS to a final concentration of 2% followed by boiling for 10 min. The denatured sample was diluted 20-fold before the addition of antibody. Immunoprecipitations performed on cells metabolically labeled with [³⁵S]methionine were analyzed by separation of immune complexes by SDS-PAGE on 8% gels at 50 mA and visualized by fluorography.

Proteolytic mapping with V8 protease

Mapping with V8 protease from *Staphylococcus aureus* was performed as described previously (Harlow and Lane 1988).

Kinase assays

Immunoprecipitations were performed on unlabeled cell lysates, and complexes were adsorbed onto protein A-Sepharose beads and washed three times in lysis buffer and once in kinase buffer containing 50 mM Tris (pH 7.4), 10 mM MgCl₂, and 1 mM DTT. Beads were resuspended in 50 µl of kinase buffer containing 1 µM ATP and 5 µCi of [γ-³²P]ATP. Reactions were incubated at room temperature for 30 min, stopped by the addition of 2× sample buffer, and resolved on a 10% polyacrylamide gel.

Preparation of whole-cell extracts

Whole-cell extracts were prepared as described previously (Raychaudhuri et al. 1987). Frozen cell pellets (washed in PBS) were thawed on ice and resuspended in 1.5 volumes of lysis buffer containing 10 mM HEPES (pH 7.6), 5 mM KCl, 1.5 mM MgCl₂, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF, and 0.5 mM Na₃VO₄. After 15 min of incubation on ice, 1.66 volume of extraction buffer [20 mM HEPES (pH 7.6), 1.6 M KCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF, 0.5 mM Na₃VO₄] was added, and extracts were rocked at 4°C for 45 min. Extracts were centrifuged at 100,000g for 45 min, and the supernatants were then taken and dialyzed for 1.5 hr at 4°C against 20 mM HEPES (pH 7.6), 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF, and 0.5 mM Na₃VO₄. Precipitates formed during the dialysis were removed by centrifugation at 10,000g for 5 min. Protein concentrations were determined by the Bradford method (Bio-Rad), and extracts were aliquoted and stored at -70°C.

Deoxycholate release from immune complexes

Immunoprecipitations were performed using 300 µg of whole-cell extract, in a buffer containing 20 mM HEPES (pH 7.4), 40 mM KCl, 1 mM MgCl₂, and 0.1 mM EDTA to which 3 mg/ml of BSA had been added. After adsorption onto protein A-Sepharose beads, the immune complexes were washed four times with the above buffer. Complexes were disrupted by incubation with 0.8% sodium deoxycholate (DOC) for 10 min on ice (Baeuerle and Baltimore 1988). NP-40 was added to a final concentration of 1.5%, and samples were then analyzed by gel retardation with a ³²P-labeled E2F oligonucleotide probe.

Gel retardation assays

Gel retardation assays were performed as described previously (Yee et al. 1987). Cell extract (5–10 µg) was used directly in a binding buffer containing 20 mM HEPES (pH 7.4), 40 mM KCl, 1 mM MgCl₂, 0.1 mM EDTA, and 0.1% NP-40, to which was added 0.5 ng of ³²P-labeled probe and 1 µg of sonicated salmon sperm DNA. For antibody shifts, gel shift reactions were incubated with 1 µl of antibody for 10 min before probe addition. For blocking, antibodies were preincubated on ice for 30 min with 10 µg of fusion protein. The reaction products were separated on a 4% polyacrylamide gel run in 0.25× TBE at 4°C at 180 V. The gel was then dried and exposed to film.

Oligonucleotides

The sequences of the oligonucleotides (Cao et al. 1992) are as follows:

E2F: ATTTAAGTTTCGCGCCCTTTCTCAA
MT: ATTTAAGTTTCGATCCCTTTCTCAA

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