Cell cycle-specific association of E2F with the p130 E1A-binding protein

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Association of the E2F transcription factor with the pRb and p107 proteins appears to regulate the activity of E2F and, in turn, affect cell cycle progression. We found, however, that pRb and p107 are only minor E2F-associated proteins in G₀/G₁ mouse fibroblasts, and we sought to identify the major E2F partner protein in these cells. Because the adenovirus E1A oncoprotein seemed able to bind to the G₀ E2F partner, we enriched for proteins that associated both with an E2F-binding site DNA column and with E1A. The major species in G₀ and early G₁ fibroblasts detected with this approach had properties identical to the pRb- and p107-related p130 protein. In serum-stimulated cells, p107 replaced p130 as the major E2F-associated protein near the G₁/S border, concomitant with an increase in p107 protein levels. p130-E2F complexes resembled p107-E2F complexes in their ability to bind to cyclin-cdk kinases, and they appeared to be associated with the cyclin E-cdk2 kinase in late G₁ cells. These observations indicate that E2F transcription factors are regulated by a succession of partner proteins with which they associate during defined stages of the cell cycle.

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E2F transcription factors are required for the regulation of a number of genes involved in cell growth control. Known E2F-responsive genes, as well as other genes with promoter E2F sites, are induced when resting G₀ cells are stimulated to reenter the cell cycle (Blake and Azizkhan 1989; Hiebert et al. 1989; Thalmeier et al. 1989; Dalton 1992; Nevins 1992; Lam and Watson 1993). Some E2F-responsive genes, such as *c-myc*, are induced during the transition from G₀ to G₁, whereas others, such as dihydrofolate reductase, are induced near the G₁/S border (Kelly et al. 1983; Farnham and Schimke 1985). Modulation of E2F function may ensure the appropriate expression of these genes during specific phases of the cell growth cycle.

The sequence-specific DNA-binding activity known as E2F consists of a group of cellular proteins, including E2F-1 and DP-1, that appear to bind as heterodimers to E2F sites in various promoters (Helin et al. 1992, 1993; Kaelin et al. 1992; Girling et al. 1993; Huber et al. 1993; Lees et al. 1993). The function of these E2Fs may be regulated in turn by their cell cycle-specific association with yet other proteins. For example, some E2Fs associate with the hypophosphorylated form of the retinoblastoma protein (pRb) found in the G₀ and G₁ phases of the cell cycle but do not associate with hyperphosphorylated ppRb found in the S, G₂, and M phases of this cycle

(Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Chellappan et al. 1991; Mittnacht and Weinberg 1991). In cells transfected with pRb expression vectors, pRb inhibits the expression of E2F-dependent genes (Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992; Zamanian and LaThangue 1992), suggesting that E2F binding is a mechanism used by pRb to regulate cell cycle progression.

E2Fs have also been found in association with the pRb-related p107 protein (Ewen et al. 1991; Mudryj et al. 1991; Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992) and have been postulated to bind a third protein distinct from pRb and p107 (Chittenden et al. 1993). Like pRb, p107 also inhibits E2F-dependent gene expression (Schwartz et al. 1993; Zamanian and La Thangue 1993; Zhu et al. 1993). The E2F species that complex with p107, however, may be distinct from those that complex with pRb (Dyson et al. 1993). p107-containing E2F complexes are first readily detected near the G₁/S border and also contain cyclin E-cdk2 or cyclin A-cdk2 kinases (Cao et al. 1992; Devoto et al. 1992; Lees et al. 1992; Pagano et al. 1992; Shirodkar et al. 1992). Thus, the different E2Fs may be controlled by specific pRb-related protein regulators at distinct points in the cell cycle.

The interactions between E2Fs and their associated proteins can be disrupted by adenovirus E1A and other DNA tumor virus oncoproteins that bind pRb and p107 and block their ability to associate with E2Fs (Bagchi et al. 1990, 1991; Chellappan et al. 1991, 1992; Mudryj et

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al. 1991; Shirodkar et al. 1992; Chittenden et al. 1993). In effect, these oncoproteins liberate E2Fs from pRb/p107 control and in this way promote the expression of E2F-dependent genes (Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992; Zamanian and LaThangue 1992, 1993; Schwarz et al. 1993).

To elucidate the role of E2Fs in cell cycle control, we have sought to identify the components of E2F-containing complexes that form at various points in the cell cycle. In the course of this work, we detected a novel E2F-containing complex that is present in the G₀ and G₁ cell cycle phases and identified an E2F-associated protein present in this complex. We explore the implications of the existence of this E2F complex for the control of E2F activity during cell cycle progression.

Results

Association of E2F with other proteins in G₀

In our initial experiments, we utilized a mobility shift assay to detect E2Fs and E2F-containing protein complexes through their ability to bind and retard the migration of a radiolabeled oligonucleotide containing an E2F-binding site.

Two distinct E2F-containing complexes were detected in serum-starved mouse embryo fibroblasts (MEFs; Fig. 1, lane 4). Both complexes were specific for the E2F-binding DNA sequence because their binding to a ³²P-labeled c-myc promoter E2F site was reduced by the addition of excess unlabeled oligonucleotide containing the two adenovirus E2 promoter E2F sites but not by an equal amount of a similar oligonucleotide having E2F site point mutations (lanes 4–6).

The faster migrating DNA–protein complex appeared to contain only free E2F because it comigrated with a complex formed by free E2F purified from HeLa cells (lanes 1,4). The second more slowly migrating complex appeared to contain E2F in association with another protein because this complex was disrupted upon incubation of the extract with either deoxycholate or E1A (data not shown), as observed previously for G₀ E2F complexes found in NIH-3T3 fibroblasts (Mudryj et al. 1991).

The behavior of the slowly migrating E2F complex in response to deoxycholate and E1A was reminiscent of pRb–E2F complexes characterized by others (Chellappan et al. 1991; Shirodkar et al. 1992). To determine whether pRb is a component of the G₀ E2F complex, wild-type MEF E2F complexes were compared with those of *Rb*[−] MEFs, the latter having been prepared from mouse embryos that had inherited two inactivated *Rb* gene copies (Jacks et al. 1992). The normal and *Rb*[−] MEF G₀ extracts contained identically migrating E2F complexes (lanes 4,8–10), indicating that such complexes do not contain pRb as a major component.

Yet other evidence distinguished the slowly migrating MEF G₀ E2F complex from pRb–E2F complexes. Thus, the G₀ E2F complex migrated more slowly than pRb–E2F complexes formed in extracts of mouse splenic lympho-

Figure 1. Characterization of E2F-containing complexes in G₀ MEFs. Extracts of wild-type (lanes 4–7) and *Rb*[−] (lanes 8–11) MEFs were incubated with a ³²P-labeled c-myc promoter E2F site and subjected to gel electrophoresis. Reactions included a 20-fold excess of unlabeled oligonucleotide containing the adenovirus E2 promoter E2F sites (lanes 5,9) or point-mutated E2F sites (lanes 6,10), or the 21C9 pRb antibody (lanes 7,11). Mobility shifts were also performed with affinity-purified HeLa cell E2F in the absence (lane 1) or presence (lanes 2,3) of affinity-purified pRb, and with nuclear extracts of primary mouse splenic lymphocytes (lanes 12,13) and U937 monocytes (lanes 14,15), with 21C9 pRb antibody added as indicated (lanes 3, 13, 15). The locations of free E2F, E2F–pRb complexes, and unidentified E2F–X complexes are indicated. All incubations included 0.4 mM spermine to facilitate detection of pRb–E2F complexes.

cytes and human U937 monocytes (lanes 8,12,14). The presence of pRb in the lymphocyte and monocyte complexes could be verified by the fact that their migration was retarded (i.e., supershifted) by a pRb monoclonal antibody (lanes 12–15). The MEF G₀ complex also migrated more slowly than pRb–E2F complexes formed in vitro by mixing purified HeLa cell E2F with purified pRb (lanes 1–4).

Although the great majority of the E2F-containing complexes in the *Rb*⁺ cell extracts appeared to lack pRb, a small amount of pRb–E2F complex could be detected in these cell extracts. Thus, a novel mobility shift complex was observed when *Rb*⁺ MEF extracts, but not *Rb*[−] MEF extracts, were incubated with pRb antibody (lanes 7,11). These results indicate that pRb is a minor component of E2F complexes in serum-starved MEFs.

Complexes of E2F with p107 in G₀ and G₁

Besides pRb, the other well-characterized E2F-binding protein in the cell is p107, which has been shown to

complex with E2F in the late G₁ and S phases of the cell cycle (Lees et al. 1992; Shirodkar et al. 1992; Schwarz et al. 1993). To test whether the observed G₀ complexes might contain p107, the MEF extracts described above and control extracts from S-phase cells were incubated with p107 antibodies (generously provided by M. Dembski and N. Dyson).

In preliminary experiments, saturating amounts of a purified p107 monoclonal antibody supershifted only a small fraction of the G₀ E2F complexes, whereas it supershifted nearly all of the S-phase E2F complexes (data not shown). The supershifted complexes induced by p107 antibody bound specifically to the oligonucleotide E2F site because they were not detected in the presence of excess E2F site-containing oligonucleotide (data not shown). In addition, a p107 polyclonal mouse antiserum had no detectable effect on G₀ complexes, but it abolished S-phase E2F complexes. The mouse antiserum probably recognizes different p107 epitopes than the monoclonal antibody because it disrupted but did not effectively supershift most p107-containing S-phase complexes (data not shown). These experiments suggested that p107 is not the major G₀ E2F-associated protein. Further experiments used the more readily synchronized A31 BALB/c 3T3 cell line to explore when p107 does become the major E2F partner during cell cycle progression.

A31 cell extracts were prepared 0, 3, 7, 11, and 15 hr after serum stimulation of quiescent A31 cells and used for mobility shift assays (Fig. 2). Whereas the mobility of E2F complexes was unchanged from 0 to 7 hr, a more slowly migrating complex was first detected in the extracts prepared at 11 hr and this complex became prominent at 15 hr (lanes 1–5), as the first cohort of these cells (5% of the total) passed from G₁ into S phase (as determined by flow cytometry analysis; data not shown). Addition of saturating amounts of p107 monoclonal antibody to extracts prepared at the 0- to 11-hr time points caused 10–15% of the E2F complexes to supershift while causing ~70% of the complexes isolated from cells at 15 hr postserum stimulation to supershift (Fig. 2, lanes 6–10). Thus, p107 appears to become the major E2F partner only as these mouse fibroblasts approach the G₁/S border.

Biochemical identification of E2F-associated proteins in extracts of G₀/G₁ mouse fibroblasts

We concluded from the above experiments that neither pRb nor p107 is the major E2F-associated protein in G₀ and G₁ mouse fibroblasts and sought to identify the apparently novel G₀/G₁ E2F partner. The ability of the adenovirus E1A protein to dissociate mouse fibroblast G₀/G₁ E2F complexes (Mudryj et al. 1991; D. Cobrinik, data not shown) suggested that E1A binds to the G₀/G₁ E2F partner in the same way that E1A binds to pRb or p107. For this reason, we designed a strategy to purify this E2F-associated protein that relied on its predicted affinity for both E2F and E1A.

In detail, the enrichment of the E2F partner involved

Figure 2. Detection of G₀ and G₁ E2F complexes that do not contain p107. Extracts were prepared from A31 cells following serum starvation (lanes 1,6) or 3, 7, 11, or 15 hr after serum stimulation (lanes 2–5,7–10) and subjected to mobility shift analysis in the absence (lanes 1–5) or presence (lanes 6–10) of SD-15 p107 antibody. The positions of free E2F, E2F complexes, and p107 antibody-dependent supershift complexes (arrow) are indicated. Cells used for this experiment were metabolically labeled with [³⁵S]methionine, and the ³⁵S signal was blocked by autoradiography film.

an initial chromatography of metabolically labeled proteins from serum-starved A31 cells over a herring sperm DNA column, followed by chromatography of this column's flowthrough fraction over an oligonucleotide column containing adenovirus E2 promoter sequences that bind E2F. Complexes adsorbing to these columns were eluted in high ionic strength buffer and mixed with E1A oncoprotein prepared from baculovirus-infected insect cells (Peeper et al. 1992). These mixtures were then immunoprecipitated with E1A antibody, and the proteins that coimmunoprecipitated with E1A were separated by SDS-PAGE. This strategy allows the detection of E1A-binding proteins that associate with any of the E2F-like proteins that can adhere to the E2 promoter E2F sites.

As shown in the mobility shift assays in Figure 3A, free and complexed E2F largely passed through the herring sperm DNA column but both forms of E2F were retained on the E2 oligonucleotide column. E2F DNA-binding activity was eluted from the E2 column as free E2F (lane 9), probably because of the disruption of the E2F complexes by the high ionic strength elution buffer.

Equal amounts of radiolabeled protein from the DNA and E2 affinity column eluates were then mixed with E1A or control insect cell lysates and immunoprecipitated with E1A antibody (Fig. 3B). Minor proteins of ~105 and 115 kD and a major protein of 126 kD were detected in the E1A coimmunoprecipitation of the E2

Figure 3. Identification of E2F-associated proteins in G₀ mouse fibroblasts using an E2F + E1A affinity enrichment procedure. (A) Mobility shift analysis of A31 fibroblast E2F complexes subjected to chromatography over herring sperm DNA (lanes 1–5) and E2 promoter oligonucleotide (lanes 6–10) columns. The E2F-containing complexes present in the load (L), flowthrough (FT), wash (W), and elution (E) fractions are displayed. Cells used for this experiment were metabolically labeled with [³⁵S]methionine, and the ³⁵S signal was blocked by autoradiography film. (B) E1A coimmunoprecipitation of proteins eluted from herring sperm DNA (lanes 1,2) and E2 promoter oligonucleotide (lanes 3–5) columns. Equal amounts of radiolabeled proteins were mixed with lysates of wild-type (lanes 1,3) or E1A-expressing (lanes 2,4,5) baculovirus-infected cells, and the mixture was immunoprecipitated with anti-E1A (lanes 1–4) or anti-IL2-receptor (lane 5) antibodies. (C) Detection of pRb, p107, and p126. Equal amounts of radiolabeled proteins eluted from herring sperm DNA (lane 1), wild-type E2F site (lanes 2,4,5), or point-mutated E2F site (lane 3) columns either were mixed with lysates of E1A-expressing baculovirus-infected cells and immunoprecipitated with E1A antibody (lanes 1–3), or were directly immunoprecipitated with pRb (lane 4) or p107 (lane 5) antibodies. The positions of molecular mass markers are indicated in kilodaltons.

column eluate (lane 4). These proteins were not detected in the absence of E1A (lane 3) nor were they seen when a control antibody was used (lane 5). Moreover, these proteins were present at far lower levels in an E1A coimmunoprecipitation of the herring sperm DNA column eluate (lane 2). Similar results were obtained with primary MEFs (data not shown). On the basis of these experiments, we concluded that the E2 oligonucleotide column retained a series of proteins that were also able to associate with E1A.

We then tested whether retention of these proteins by the E2 oligonucleotide column depended on the presence of the E2F recognition sequence. Thus, E2F complexes that flowed through the herring sperm DNA column were chromatographed subsequently over columns consisting of either an E2 promoter E2F recognition sequence oligonucleotide or a point-mutated version thereof that is not bound effectively by E2F (Hiebert et

al. 1989). Following coimmunoprecipitation of the proteins eluted from these columns in the presence of E1A, we found that the 105-, 115-, and 126-kD species were efficiently retained by the wild-type oligonucleotide column but were not retained by the column bearing the mutant oligonucleotide or by the herring sperm DNA column (Fig. 3C, lanes 1–3). We concluded that these species associate with proteins that have the identical DNA recognition sequence as E2F transcription factors.

The electrophoretic mobilities of the two more rapidly migrating E2F + E1A-associated proteins were reminiscent of those ascribed to pRb and p107. To determine whether these E2F-associated proteins were pRb and p107, the eluate of the wild-type E2F site column was immunoprecipitated with pRb and p107 antibodies. pRb antibody immunoprecipitated a 105-kD protein that comigrated with the E2F + E1A-associated 105-kD protein (Fig. 3C, lane 4), whereas p107 antibody immuno-

precipitated a 115-kD protein that comigrated with the E2F+E1A-associated 115-kD protein (Fig. 3C, lane 5). Thus, E2F is associated with pRb, p107, and a third predominant partner protein—provisionally termed p126—in G₀ mouse fibroblast extracts.

Relationship between p126 and the E1A-associated p130 protein

The apparent size of p126 was similar to that of the E1A-associated p130 protein detected by virtue of its ability to be coimmunoprecipitated with E1A from lysates of adenovirus-transformed human embryonic kidney 293 cells (Harlow et al. 1986). A series of experiments comparing the electrophoretic migration rates, phosphatase sensitivities, antibody reactivities, and proteolytic peptide maps of the two proteins led us to believe that p126 was a modified form of this p130. These experiments utilized human cells, instead of the mouse fibroblasts used earlier, to control for any species-specific size or sequence differences.

We first compared the electrophoretic migration of 293 cell p130 to that of E2F+E1A-associated proteins in several human cell types (Fig. 4). A 126-kD E2F+E1A-associated protein detected in serum-starved human SiFt fibroblasts comigrated with mouse fibroblast p126 (data not shown) but migrated more rapidly than 293 cell p130 (lanes 1,2). In contrast, distinct E2F+E1A-associated proteins comigrated with both p126 and p130 in serum-starved human HaCaT keratinocytes and embryonic kidney (HEK) and retinal (HER) cells (lanes 3–5). Like pRb and p107, the 126- and 130-kD proteins required E1A for their coimmunoprecipitation with E1A antibody (lanes 6–9) and were more effectively retained on the E2 column than on the herring sperm DNA column (lanes 10–13). Each of these proteins was also retained on the wild-type but not the point-mutated E2F site column (data not shown). Thus, in addition to p126, an E1A-binding pro-

tein identical in size to p130 associates with E2F in certain cell types.

We then tested whether p130 and the E2F+E1A-associated 126- and 130-kD species might be differentially phosphorylated forms of the same protein. ³⁵S-Labeled cellular E2F+E1A-associated proteins and 293 cell E1A-associated proteins were treated with potato acid phosphatase or with phosphatase buffer before electrophoresis (Fig. 5A). Phosphatase converted the 293 cell p130 and the HEK, HER, and HaCaT cell 130-kD protein bands to 126-kD species (lanes 1,2,5,6; data not shown), whereas sodium phosphate, an inhibitor of this phosphatase, prevented these conversions (data not shown). Phosphatase treatment also caused a modest narrowing of the SiFt 126-kD protein band that comigrates with phosphatase-treated p130 (lanes 3,4). These results support the view that p130 and the E2F+E1A-associated 126- and 130-kD species are differentially phosphorylated forms of the same protein.

To determine whether the 126- and 130-kD E2F+E1A-associated proteins are both recognized by a p130 antibody, ³⁵S-labeled HaCaT cell E2F+E1A-associated proteins were separated by electrophoresis, transferred to nitrocellulose, and either probed with p130 antibody or detected directly by autoradiography (Fig. 5B). The p130 antibody recognized 126- and 130-kD proteins, as well as the p107 protein with which it cross-reacts (Li et al. 1993), in anti-E1A immunoprecipitates of the E2 column eluate performed in the presence but not in the absence of E1A (lanes 2,3). This antibody failed to recognize such proteins in immunoprecipitates of the herring sperm DNA column eluate (lane 1). The 126- and 130-kD proteins recognized by the p130 antibody comigrated with ³⁵S-labeled E2F+E1A-associated proteins that had been transferred to the same membrane (lane 5), and the 130-kD protein was largely converted to 126-kD by phosphatase treatment (lane 4). The recognition of both the 126- and 130-kD E2F+E1A-associated proteins by p130

Figure 4. Comparison of cellular E2F+E1A-associated proteins with 293 cell E1A-associated proteins. Whole cell extracts prepared from metabolically labeled serum-starved human SiFt fibroblasts, HaCaT keratinocytes, HEK, or HER cells were subjected to chromatography over herring sperm DNA (lanes 10–13) and E2 promoter oligonucleotide (lanes 2–9) columns. Equal amounts of radiolabeled proteins eluted from these columns were mixed with lysates of E1A-expressing (lanes 2–5,10–13) or wild-type (lanes 6–9) baculovirus-infected cells, and the mixtures were immunoprecipitated with E1A antibody. Lane 1 displays metabolically labeled 293 cell proteins coimmunoprecipitated with E1A antibody. The pRb, p107, p130, and p300 species and the positions of molecular mass markers are indicated.

Figure 5. Relationship between p130 and the 126- and 130-kD E2F + E1A-associated proteins. (A) Phosphatase sensitivity. Metabolically labeled 293 cell E1A-associated proteins (lanes 1,2) and SiFt (lanes 3,4) and HEK (lanes 5,6) E2F + E1A-associated proteins were treated with potato acid phosphatase (lanes 2,4,6) or phosphatase buffer (lanes 1,3,5) before electrophoresis. The 126- and 130-kD E2F + E1A-associated proteins (arrows) and the positions of molecular mass markers are indicated. (B) Recognition of the E2F + E1A-associated 126- and 130-kD proteins by p130 antibody. Extracts of serum-starved HaCaT cells were subjected to chromatography over herring sperm DNA (lane 1) and E2 promoter oligonucleotide (lanes 2–5) columns. Equal amounts of radiolabeled proteins eluted from these columns were mixed with lysates of E1A-expressing (lanes 1,3,4,5) or wild-type (lane 2) baculovirus-infected cells, the mixtures were immunoprecipitated with E1A antibody, and the immunoprecipitates were incubated with potato acid phosphatase (lane 4) or phosphatase buffer (lanes 1–3,5). Proteins were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane that was then subjected either to Western blotting with a p130 monoclonal antibody (lanes 1–4) or to autoradiography (lane 5). The positions of molecular mass markers are indicated. (C) Peptide maps of p130 and the E2F + E1A-associated 130- and 126 kD-proteins. 293 cell E1A-associated proteins and HaCaT cell E2F + E1A-associated proteins were separated by polyacrylamide gel electrophoresis, and gel slices containing 293 cell p130 (lanes 1–3) and HaCaT cell 130-kD (lanes 4–6) and 126-kD (lanes 7–9) proteins were incubated with 0.3, 1.5, or 7.5 μ g of a crude trypsin preparation as indicated. Similarly treated pRb is shown as a control (lanes 10–12). The positions of molecular mass markers are indicated in kilodaltons.

antibody supports the notion that these species are differentially phosphorylated forms of p130.

The relatedness of the 126- and 130-kD E2F-associated proteins and p130 was solidified by comparing the sizes of proteolytic digestion products of these species. The peptide maps produced by the digestion of HaCaT cell 126- and 130-kD E2F + E1A-associated proteins with a crude trypsin preparation were identical to those of 293 cell p130 but differed from a map of pRb included as a control (Fig. 5C). These experiments show conclusively that two differentially phosphorylated forms of the previously identified p130 E1A-binding protein associate with E2F transcription factors.

Cell cycle-dependent association of E2F with p130

The E2F + E1A affinity purification scheme described

above was used to survey the changing spectrum of E2F partners as the cell moves from G_0 through G_1 and into S. For this experiment, we analyzed the extracts from serum-starved and restimulated A31 cells that had been used in the study described in Figure 2. These extracts had been labeled with [35 S]methionine, either for 4 hr before restimulation, in the case of the G_0 preparation, or for the final 2 hr of each of the restimulation periods.

The faster migrating form of p130 was the major E2F + E1A-associated species labeled in G_0 and 3 hr after serum stimulation, whereas p107 became the major E2F partner detected as the cells approached the G_1 /S border 11–15 hr after serum stimulation (Fig. 6A, lanes 1–6). Although the E2F + E1A-associated p130 appeared to be reduced 7 hr after serum stimulation, p130 is likely to continue to be the major E2F partner detected by mobility shift analysis at this time (see Fig. 2), because only

Figure 6. Cell cycle-dependent association of E2F with p130 and p107. (A) Transitions between E2F-associated proteins in A31 cells. Metabolically labeled A31 cell proteins were subjected to chromatography over herring sperm DNA (lanes 7–11) and E2 promoter oligonucleotide (lanes 1–6) columns, and equal amounts of radiolabeled proteins eluted from these columns were coimmunoprecipitated with E1A. A31 cells were serum starved (lanes 1,7) or restimulated for 3, 7, 11, or 15 hr as indicated (lanes 2–5,8–11). The eluate of the E2 promoter oligonucleotide column corresponding to 15 hr serum-stimulated cells was also directly immunoprecipitated with p107 antibody (lane 6). The positions of molecular mass markers are indicated in kilodaltons. (B) Induction of p107 synthesis in serum-stimulated A31 cells. Equal amounts of radiolabeled protein prepared from metabolically labeled serum-starved (lanes 1–6) or restimulated (lanes 2–5,7–10) cells were immunoprecipitated with anti-p107 (lanes 1–5) or anti-E1A control (lanes 6–10) antibodies. (C) Induction of p107 protein levels in serum-stimulated A31 cells. Equal amounts of protein prepared from serum-starved (lane 1) or restimulated (lanes 2–5) cells were immunoprecipitated with p107 antibody, followed by detection of the immunoprecipitated p107 by Western analysis.

newly synthesized p130 is detected with the affinity enrichment procedure. The p130 and p107 proteins detected in these assays were effectively retained on the E2 affinity but not on the herring sperm DNA columns (Fig. 6A, lanes 7–11). Protein with the approximate migration of pRb was detected in only small amounts.

Figure 6A shows a dramatic increase in E2F-associated ^{35}S -labeled p107 between 7 and 15 hr. This was paralleled by an increase in the quantity of ^{35}S -labeled p107 that could be immunoprecipitated from extracts of metabolically labeled A31 cells with p107 antibody (Fig. 6B) and by a similar increase in total p107 levels detected by Western analysis of p107 immunoprecipitates (Fig. 6C). Taken together with the mobility shift assays in Figures 1 and 2, our results indicate that p130 is the major E2F-associated protein in G_0 and early G_1 mouse fibroblasts and that p107 only becomes the major E2F partner when its levels increase near the G_1/S border.

Interaction of the mouse fibroblast p130–E2F complex with cyclins and cdk2

Close examination of our initial data revealed that a portion of the E2F complexes detected in late G_1 , 11 hr after serum stimulation, migrated with rates reminiscent of the S-phase E2F–p107–cyclin A–cdk2 complex but appeared to lack p107 (Fig. 2, lanes 4,9). Thus, we speculated that the p130–E2F complexes that predominate in G_0 and G_1 cells form higher order complexes with cyclins and their associated kinases in late G_1 . As one test of this possibility, we gauged the ability of cyclins and cdk2 prepared from baculovirus vector-infected insect cells to interact with MEF G_0 E2F complexes, which

contain p130 as the major E2F-associated protein (Fig. 7A).

Addition of either cyclin E- or cyclin A-containing insect cell lysates retarded the migration of a minority of the G_0 E2F complexes, both in the absence and presence of cdk2-containing lysates (lanes 1–3,7–9). The addition of cyclin E- or cyclin A-containing lysates with cdk2-containing lysates, however, retarded the migration of nearly all of the G_0 E2F complexes (lanes 4,10). Addition of cdk2-containing lysates alone had no effect on the G_0 E2F complexes (lane 13), whereas uninfected cell lysates or cyclin B-containing lysates in combination with either cdk also failed to retard these complexes (data not shown). The cyclin–cdk2-dependent complexes migrated similarly to p107-containing S-phase complexes, but the majority of these reconstituted complexes appeared to contain p130, and not p107, because they were not affected by p107 monoclonal antibody or by anti-p107 mouse serum (data not shown).

The cyclin–cdk2-dependent E2F complexes could be disrupted by the appropriate anti-cyclin antiserum and by cdk2 antibody but not by pre-immune or control antisera (lanes 4–6,10–12,16,17). cdk2 antibody additionally disrupted the complexes formed with G_0 extract and cyclin A-containing lysate added alone (lanes 18,19) but had no effect on G_0 E2F complexes formed in the absence of added proteins (lanes 14,15). Thus, the small amount of the G_0 complex that could be shifted by the addition of a cyclin (lanes 2,8) apparently utilized cdk2 molecules that were already present in the G_0 extract.

Together, these data indicated that the G_0 E2F-associated protein identified as p130 by the E2F + E1A affinity enrichment procedure is similar to p107 in its ability to bind simultaneously E2F and a cyclin–kinase complex.

Figure 7. Interactions between p130-E2F complexes and cyclin-cdk kinases. (A) Interactions between MEF p130-E2F complexes and cyclin-cdks expressed in baculovirus vector-infected cells. MEF extracts were incubated with lysates of baculovirus-infected cells expressing cyclin E (lanes 2–6) cyclin A (lanes 8–12,16–19), cdk2 (lanes 3,9) or cdk2 (lanes 4–6,10–13,16,17) in mobility shift reactions. Incubations were subsequently supplemented with cyclin E antiserum (lane 5), a corresponding preimmune serum (lane 6), cyclin A antiserum (lane 11), a normal rabbit serum (lane 12), or cdk2 antibody (lanes 15,17,19); and the reaction contents were separated by electrophoresis. The positions of E2F and p130-E2F (E2F-X) complexes are indicated. (B) Cell cycle-dependent interaction between the p130-E2F complex and the cyclin E-cdk2 kinase. Extracts (15 μ g) prepared from A31 cells that were serum-starved (lane 1) or restimulated for 8, 9, 10, or 11 hr (lanes 2–9, as indicated) were incubated in mobility shift reactions with saturating amounts of SD-15 p107 antibody (lanes 1–9), cyclin E antiserum (lane 7), the corresponding preimmune serum (lane 8), and cdk2 antibody (lane 9). The positions of free and complexed E2F are indicated on the left. The position of a p107 antibody-dependent E2F complex is indicated by the arrow.

These higher order complexes are dependent on the presence of both the cyclin and cdk subunit, similar to the association of cyclin-cdks with p107 or a G₁ E2F complex (Peeper et al. 1993; Schwarz et al. 1993).

Because cyclin E is first found in substantial amounts in the late G₁ phase of the cell cycle (Lew et al. 1991), when slowly migrating E2F complexes lacking p107 appear, we tested whether these late G₁ complexes contain cyclin E and cdk2. As shown in Figure 7B, slowly migrating E2F complexes that were resistant to saturating

amounts of p107 antibody were formed from 8 to 11 hr after serum stimulation (lanes 1–5). We found that antiserum directed against cyclin E disrupted the complexes detected 9 hr after serum stimulation, whereas the corresponding preimmune serum had no effect (lanes 6–8). Similarly, anti-cdk2 antibody selectively disrupted this slowly migrating species (lane 9).

These results indicate that E2F and its associated p130 protein not only form higher order complexes with exogenous, baculovirus-expressed cyclins and cdks but also

form such higher order complexes with cyclins and cdk2 expressed in late G₁ cells. Importantly, the present experiments do not indicate whether these complexes are actually present throughout late G₁. Thus, such quaternary complexes might only form in the narrow window of time after the induction of high levels of cyclin E expression and before p107 becomes the predominant E2F-associated protein.

Discussion

The abilities of pRb and p107 to bind E2F transcription factors and to inhibit the expression of E2F-dependent genes suggested that these E2F interactions play important roles in cell growth control (Nevins 1992). Here, we show that E2Fs are also bound by the p130 protein. p130 was discovered as a prominent E1A-associated cellular protein (Harlow et al. 1986), and has recently been found to be structurally related to pRb and p107 (Hannon et al., Li et al., both this issue). Our present finding that p130 binds to E2Fs indicates that p130 also has functional analogies with pRb and p107.

The mobility shift and affinity enrichment procedures used here demonstrate that p130 is the major E2F-associated protein in G₀ A31 fibroblasts. p130 also appeared to be the major E2F-associated protein in G₁ fibroblasts that were released from serum starvation, whereas only small amounts of pRb and p107 associated with E2Fs in extracts prepared from these serum-starved or restimulated cells. In a related study, Chittenden et al. (1993) have found that the major E2F complex in quiescent and stimulated G₁ lymphocytes appears to contain neither pRb nor p107. Thus, p130 may be a component of the major G₀/G₁ E2F complex in lymphocytes, as well as in fibroblasts. However, pRb, p107, and p130 were associated with E2Fs in comparable amounts in other types of serum-starved cells, such as HaCaT keratinocytes.

The different patterns of E2F-associated proteins observed in these serum-starved cell types could reflect cell type-specific differences in the expression of the various E2Fs or the pRb-related E2F partners (Bernards et al. 1989; Helin et al. 1992; Szekely et al. 1992; Slack et al. 1993). Alternatively because nearly 100% of our serum-starved A31 cells were in the G₀/G₁ phase of the cell cycle, whereas only 94% of HaCaT cells were in G₀/G₁ after a much longer period of serum starvation (data not shown), p130 may become the predominant regulator of E2F only in cells that are effectively growth-arrested in G₀ in response to mitogen deprivation.

Despite our finding that p130 is the predominant E2F partner in mouse fibroblasts, p130 might not regulate all E2Fs present in such cells. This is because the DNA-binding activity termed E2F is composed of a group of distinct polypeptides (Helin et al. 1992; Kaelin et al. 1992; Girling et al. 1993; Huber et al. 1993; Lees et al. 1993) that may each associate specifically with only a subset of the three pRb-related proteins. Such a notion is supported by the recent finding that pRb and p107 associate with different forms of E2Fs (Dyson et al. 1993) and by evidence of apparently distinct species of E2Fs in cer-

tain lymphocyte multiprotein complexes (Chittenden et al. 1993). The ability of E2F family members to interact with distinct pRb-related regulators may allow the various E2F subtypes and E2F-responsive genes to be controlled independently during the cell cycle. In this context, two or three pRb-related proteins might be required concurrently for normal E2F regulation.

Of the three E2F-associated proteins, p107 and p130 are by far the most closely related (Hannon et al., Li et al., both this issue). This raises the possibility that p107 and p130 interact with the same E2F species and that the dramatic increase in E2F-associated p107 near the G₁/S border results primarily from a similarly dramatic increase in p107 protein levels shown here. The distinct cell cycle-dependent patterns of E2F association exhibited by p130 and p107, however, could also reflect the availability of specific E2Fs with which p130 and p107 interact, or processes that regulate the expression or the E2F-binding capability of p130.

Regardless of the specific E2F subtypes with which p130 and p107 interact, our data and that of others indicate that E2F complexes containing either of these proteins can associate with cyclinE-cdk2 and cyclin A-cdk2 kinases. Although the function of the cyclin-cdk2-containing higher order E2F complexes is not known as yet, the similarities between p130 and p107 suggest that p130- and p107-containing complexes may serve the same function, despite their differential regulation during the cell cycle. Higher order p107-containing complexes were detected previously in late G₁- and S-phase cell extracts (Cao et al. 1992; Devoto et al. 1992; Lees et al. 1992; Shirodkar et al. 1992), and we demonstrate here that p130-E2F-cyclin E-cdk2 complexes form in late G₁ before the induction of high levels of p107. Our data suggest that higher order complexes containing p130 or p107 may be present in cells continuously from late G₁ into S phase.

Others have shown that mutant forms of the E1A oncoprotein that bind pRb and p107, but are severely impaired in p130 binding, exhibit a reduced ability to transform primary cells in cooperation with an activated *ras* oncogene (Whyte et al. 1988; Giordano et al. 1991). This suggests that p130 plays an important role in the regulation of cell proliferation and, further, that it may function as a suppressor of cell transformation. The present results provide one mechanistic explanation of how p130 may participate in such control. By regulating E2F function, p130 may indirectly control the expression of critical E2F-responsive genes in G₀ and early G₁ and in this way program early steps in the cell growth cycle. For other E2F-responsive genes, or in other cell growth stages, such control may be exerted by the p130 cognates pRb and p107, in this way ensuring an orderly regulation of the transcription of cell growth-promoting genes.

Materials and methods

Cells and cell culture

MEFs were prepared from 12.5-day wild-type and *Rb*^{-/-} mouse embryos (Jacks et al. 1992) as described (Robertson 1987). Early

passage A31 BALB/c 3T3 cells were obtained from C. Stiles (Dana Farber Cancer Center, Boston, MA). HEK and 293 cells were obtained from A. Van der Eb. HER cells were provided by A. Van der Eb and by D. Alberts (Massachusetts General Hospital, Boston, MA). HaCaT cells (Boukamp et al. 1988) were a gift from P. Boukamp. SiFt fibroblasts were obtained from J. Rheinwold. U937 cells were from J. Minowada (Roswell Park Memorial Institute, Buffalo, NY). Mouse splenic lymphocytes were prepared from freshly dissected spleens.

MEFs were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO) with 10% fetal calf serum (FCS, Hyclone Laboratories). A31 and 293 cells were grown in DMEM with 10% calf serum (CS, GIBCO). SiFt, HER, and HEK cells were grown in DMEM with 15% FCS (GIBCO). HaCaT cells were grown in α minimal essential medium (α MEM, GIBCO) with 10% FCS. U937 cells were grown in RPMI (GIBCO) with 10% FCS.

Cell synchronization

MEF and A31 cells were plated at 7.5×10^5 cells per 10-cm-diam. culture dish, allowed to grow for 24 hr, and then incubated in DMEM plus 0.5% serum for 54 or 44 hr, respectively. All other cells were plated at 1×10^6 cells per 10-cm dish, allowed to grow for 48 hr, and incubated in α MEM for 72 hr (HaCaT cells) or in DME plus 0.5% FCS for 48 hr (SiFt, HEK, and HER cells).

Metabolic labeling

Cells were grown for 20 min in methionine-free DMEM (GIBCO) plus 0.5% or 10% dialyzed CS or FCS (depending on the cell type and desired growth conditions), followed by incubation in medium containing 0.15–0.3 mCi/ml of Trans³⁵S-label (ICN) for 6 hr unless otherwise indicated.

Extract preparation

Whole cell extracts were prepared from trypsinized cells at 4°C. Cells were suspended in DMEM plus 10% CS, washed in phosphate-buffered saline [PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM NaPO₄ (pH 7.0)], resuspended in 5 packed cell volumes of buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml of leupeptin, 2 μ g/ml of aprotinin, 1 mM sodium orthovanadate)], pelleted in an Eppendorf centrifuge at 6000 rpm, resuspended in 3 packed cell volumes of buffer A, homogenized in a Dounce apparatus using 100 strokes of the A pestle, and centrifuged at 14,000 rpm for 5 min. The supernatant (cytoplasmic extract) was removed and the pellet was suspended in 2 volumes of buffer A [but containing 0.4 M NaCl, 25% glycerol, and HEPES (pH 7.9) at 20 mM], incubated with mixing for 30 min, and centrifuged at 14,000 rpm for 5 min. The supernatant (nuclear extract) was combined with the cytoplasmic extract, centrifuged at 14,000 rpm for 1 min, and stored at –80°C.

Cell lysates used for immunoprecipitations were prepared by washing cells with PBS, incubating in $1 \times$ ELB [0.25 M NaCl, 0.1% NP-40, 25 mM HEPES (pH 7.0), 2.5 mM EDTA, 0.5 mM DTT, and inhibitors as above] with mixing on ice for 30 min, and centrifuging at 14,000 rpm for 5 min.

Mobility shift assays

Mobility shift assays (Kovesdi et al. 1986) utilized a gel-purified, ³²P-end-labeled double-stranded oligonucleotide probe of plus strand sequence: 5'-GGCTTCTCAGAGGCTTGGCGGAA-

AAAGAACGGACGGAGGGAT-3', representing the –82 to –40 sequence of the human *c-myc* P2 promoter. Cell extracts were incubated for 20 min at room temperature in 10- μ l reactions containing 60 μ g/ml of boiled and sonicated salmon sperm (SS) DNA, 20 mM HEPES (pH 7.6), 4% Ficoll, 2.5 mM MgCl₂, 40 mM KCl, 0.1 mM EGTA, 0.5 mg/ml of acetylated bovine serum albumin (BSA, New England Biolabs) 0.5 mM DTT, and 0.5 pmol/ml of probe, followed by electrophoresis in a 4% polyacrylamide gel in 22 mM Tris, 22 mM boric acid, and 0.6 mM EDTA.

Competitive binding reactions included a 20-fold molar excess of an unlabeled double-stranded oligonucleotide containing nucleotides –71 to –27 of the adenovirus E2 promoter and flanked by unrelated restriction sites of sequence 5'-CGCGTG-GATCCAGTTTTCGCGCTTAAATTTGAGAAAGGGCGCG-AACTAGTCCTTAC-3' for the plus strand. The corresponding point-mutated oligonucleotide substituted the underlined C residue with A and substituted the underlined G residue with T (Hiebert et al. 1989).

Protein preparations

Purified pRb and cell lysates containing cyclin A, cyclin B, cyclin E, cdk2, and cdc2 were prepared from insect cells infected with the corresponding recombinant baculoviruses as described (Desai et al. 1992; Dowdy et al. 1993; S. Mittnacht and S.F. Dowdy, unpubl.). E1A was prepared from baculovirus-infected insect cells (Peeper et al. 1992) by lysis in 2 packed cell volumes of $1 \times$ ELB for 15 min. E2F was affinity purified as described (Yee et al. 1989).

Antibodies

p107 monoclonal antibody (SD-6, SD-9, and SD-15) hybridoma tissue culture supernatants (Dyson et al. 1993) were generously provided by M. Dembski and N. Dyson. Affinity-purified cdk-2 monoclonal antibody (Tsai et al. 1993) was a gift of L.-H. Tsai and E. Harlow. Cyclin E rabbit antiserum and preimmune serum (Dulic et al. 1992) were generously provided by V. Dulic and S. Reed. Cyclin A rabbit antiserum (Pines and Hunter 1990) was the kind gift of J. Pines and T. Hunter. Normal rabbit serum and rabbit anti-mouse IgG were from Sigma. E1A monoclonal antibody M73 (Harlow et al. 1985) was an ascites preparation. pRb monoclonal antibody 21C9 (Templeton and Weinberg 1991) was a hybridoma tissue culture supernatant, and pRb monoclonal antibody G3-245 were from PharMingen. p130 monoclonal antibody Z11 (Li et al., this issue) was a hybridoma tissue culture supernatant.

The p107 SD-15 antibody used in mobility shift experiments was purified by using an anti-mouse IgG column (Sigma) as described (Harlow and Lane 1988), and the antibody was concentrated using a microcon apparatus (Amicon). In titration experiments utilizing G₀- and S-phase whole cell extracts, half-maximal supershifting of p107-containing complexes was obtained with 25 ng of purified antibody, and maximum supershifting was obtained with 75 ng (data not shown). To each mobility shift reaction 225 ng of antibody was added to ensure saturation binding to p107.

Affinity chromatography

The herring sperm DNA column was prepared by linking sheared herring sperm DNA (Sigma) to Affi-gel 10 (Bio-Rad Laboratories). Oligonucleotide columns were prepared as described (Kadonaga and Tjian 1986) using CNBr-activated Sepharose 4B (Pharmacia). The E2 oligonucleotide column consisted of mul-

timers of sequences from -26 to -81 of the adenovirus 5 E2 promoter (Imperiale and Nevins 1984) flanked by *HindIII* overhangs: 5'-AGCTTGAGATGACGTAGTTTTCGCGCTTAAATTTGAGAAAGGGCGGAACTAGTCCTTA-3' on the plus strand. The wild-type and mutant E2F site oligonucleotide columns were prepared in parallel and contained nearly identical amounts of multimerized oligonucleotide of sequences from -53 to -72 of the adenovirus 5 E2 promoter and flanked by *HindIII* overhangs: 5'-AGCTAGTTTTCGCGCTTAAATTT-3' and 5'-AGCTAGTTTTCGAGCTTAAATTT-3' for the plus strands of the wild-type and mutant columns, respectively.

Chromatography was performed using 0.5 - 0.8 ml of whole cell extract brought to 0.1% NP-40 and 600 µg/ml of boiled and sonicated salmon sperm DNA that had been passed 20 times through 23-gauge and then 27-gauge needles. The solution was stored on ice for 15 min and then loaded onto a 0.5-ml herring sperm DNA column equilibrated with buffer C [0.1 M KCl, 20 mM HEPES (pH 7.6), 0.2 mM EGTA, 2 mM MgCl₂, 0.1% NP-40, 0.5 mM DTT, and inhibitors as above]. The peak flow-through fraction of 1.3× the load volume was collected, the column was washed with 5 volumes of buffer C, and then eluted with 2× ELB [0.5 M NaCl, 0.2% NP-40, 50 mM HEPES (pH 7.0), 5 mM EDTA, 0.5 mM DTT, and inhibitors as above], initiating collection of 0.7-ml elution fractions after loading 0.5 column volumes of 2× ELB. The same procedure was used for chromatography of the herring sperm DNA column flowthrough fraction over oligonucleotide columns. The protein radioactivity content of elution fractions was determined by trichloroacetic acid precipitation. Elution fractions were brought to 0.1 mg/ml of BSA before storing at -80°C, and were brought to 250 mM NaCl by mixing with 0.33 volumes of H₂O before use in immunoprecipitation experiments.

Immunoprecipitations

E1A coimmunoprecipitations were performed by mixing affinity column eluates (0.5 × 10⁶ to 2.0 × 10⁶ cpm of radiolabeled protein) with 2 µl of E1A or control lysate, 30 µl of a 10% solution of zysorbin (Zymed Laboratories), and 1× ELB added to 0.6 ml. Solutions were mixed for 30 min at 4°C and centrifuged. The supernatants were added to 1 µl of M73 anti-E1A or control ascites, kept on ice for 1 hr, mixed with protein A-agarose (Bio-Rad Laboratories) for 1 hr, and the protein A-agarose was then washed four times in 1× ELB and prepared for electrophoresis in 6% SDS-polyacrylamide gels and fluorography (Harlow and Lane 1988). pRb and p107 were immunoprecipitated using a combination of 100 µl of 21C9 and 3 µl of G3-245 for pRb and a combination of 50 µl each of SD-6, SD-9, and SD-15 for p107.

Western analysis

³⁵S-Labeled HaCaT whole cell extract (0.5 ml) and unlabeled extract (3.0 ml) were prepared in parallel, combined, divided into four fractions of 2.75 mg, and chromatographed over four 0.75-ml herring sperm DNA columns, and the flowthrough fractions were chromatographed over 0.75-ml E2 oligonucleotide columns. The elution fractions from each E2 column were combined, brought to 0.25 M NaCl, divided into four equal fractions, and coimmunoprecipitated with E1A. Precipitated proteins were electrophoretically separated and transferred to a nitrocellulose membrane. A portion of the membrane was directly autoradiographed. The remaining membrane was incubated at 23°C in 0.05% Tween-20 in PBS (TPBS) containing 5% dried milk for 30 min and in Z11 p130 antibody diluted 0.1× in TPBS containing 2.5% dried milk for 10 hr, and was then washed and

detected using horseradish peroxidase-linked rabbit antimouse IgG and an enhanced chemiluminescence kit (Amersham).

The p107 Western blot utilized 5 mg of A31 cell extract that was immunoprecipitated with a cocktail of p107 antibodies as described above, and subjected to Western analysis, using SD-9 as the primary antibody.

Peptide mapping

E2 column eluate (1.5 × 10⁷ cpm) was coimmunoprecipitated with E1A and loaded into each of three lanes before separation in a 6% polyacrylamide gel. Gels were exposed to film for 48 hr, and protein bands were excised, treated with the indicated amounts of porcine trypsin 1 : 250 (Sigma) during electrophoresis (Cleveland et al. 1977), separated in a 16.5% SDS-polyacrylamide gel, and prepared for fluorography.

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