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p21 Disrupts the Interaction between cdk2 and the E2F-p130 Complex

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In nonproliferating or growth-arrested cells, the transcription factor E2F remains bound to the retinoblastoma-related protein p130. Accumulation of this E2F-p130 complex correlates with an arrest of the cell cycle progression. Progression through G_1 phase is associated with a cyclin-dependent binding of the cyclin-dependent kinase cdk2 to the E2F-p130 complex. By fractionating mouse L-cell extracts, we have obtained a partially purified preparation of the E2F-p130 complex that also contains cdk2. Incubation of this complex with recombinant p21 results in a disruption of the interaction between cdk2 and the E2F-p130 complex. We have also analyzed the interaction between cdk2 and the E2F-p130 complex in extracts of a cell line that expresses a temperature-sensitive mutant of p53. Incubation at the permissive temperature (32°C) results in an induction of p21 synthesis. An increase in the level of p21 in these cells correlates with a loss of cdk2 from the cdk2-containing E2F-p130 complex. We also show that the expression of a reporter gene containing E2F sites in the promoter region is reduced by the coexpression of p21. Since p21 is believed to be a mediator of p53, we speculate that the p21-mediated disruption of the cdk2-containing E2F-p130 complex plays a role in the growth suppression function of p53.

The transcription factors of the E2F family play a central role in cell proliferation. Several growth-associated genes, including c-*myc*, n-*myc*, B-*myb*, *cdc2*, *dhfr*, and the thymidine synthetase, thymidine kinase, and DNA polymerase α genes, contain E2F-binding sites in their regulatory regions (6, 12, 29, 33, 50, 55, 56, 66, 70). Some of these genes have been shown to depend on E2F for their cell cycle-regulated expression (6, 12, 34, 38, 43, 49, 53, 64). The activity of E2F is inhibited by proteins of the retinoblastoma family, such as the retinoblastoma tumor suppressor protein (pRB), p107, and p130. Inhibition of E2F activity coincides with a suppression of cell growth by these retinoblastoma family proteins (2, 3, 4, 5, 7, 8, 10, 11, 14, 23, 31, 32, 44, 45, 57, 59, 63, 67, 68, 71, 73, 75).

The tumor suppression function of pRB also appears to correlate with its ability to repress E2F-dependent transcription. The naturally occurring loss-of-function mutants of pRB are unable to regulate E2F (2, 31, 32, 57). Recent studies indicated that pRB, p107, and p130 suppress cell growth by biochemically distinct pathways involving different members of the E2F family. pRB regulates E2F1, E2F2, and E2F3, whereas p107 and p130 regulate E2F4 and E2F5 (17, 20, 27, 28, 39, 42, 46, 59, 61, 67, 73). The E2F-regulatory function and growth suppression function of pRB are inactivated by cyclin–cyclin-dependent kinase (cdk)-dependent phosphorylation of pRB (8, 9, 13, 16, 36). p107 and p130 also associate with cyclin-cdk2 complexes (7, 11, 14, 19, 24, 45, 47, 48, 63, 73). Recent experiments indicated that phosphorylation of p107 by a cyclin-cdk complex could regulate its interaction with E2F (74).

p107 has been shown to interact with cyclin A-cdk2 through sequences that are distinct from those required for binding to E2F (65, 74). This finding is also consistent with the observation that a four-member complex, E2F-p107-cyclin A-cdk2, can be detected in the extracts of cycling cells (14, 52, 60, 63). Although the function of the four-member complex is not completely understood, recent studies by Zhu et al. suggest that this higher-order complex may be an intermediate that eventually leads to a disruption of the interaction between E2F and p107 (74). Zhu et al. also identified a region in the p107 polypeptide (between residues 651 and 669) that is essential for binding to the cyclin-cdk2 complex (74). These authors also show that this region in p107 is highly homologous to the region in p21 between residues 16 and 28 (74). In addition, p21 competes with p107 for an interaction with the cyclin-cdk2 complex and dissociates the interaction between cyclin-cdk2 with the E2F-p107 complex.

p130 is structurally very similar to p107. Like p107, p130 interacts with E2F and cyclin-cdk2 (11, 24, 47, 67). In resting primary human T cells and in quiescent mouse fibroblasts, E2F remains associated with p130 in the form of a two-member E2F-p130 complex (11, 67). Stimulation of quiescent cells by serum or mitogen leads to the binding of cdk2 to the E2F-p130 complex in a cyclin-dependent manner (11). To study the cyclin-cdk2-containing E2F-p130 complex, we have fractionated mouse L-cell extracts and partially purified a complex that contains E2F, p130, and cyclin-cdk2. This complex copurifies with the E2F-p107-cyclin A-cdk2 complex through heparin-agarose and Q-Sepharose chromatographies. Using a preparation that is enriched for the p130 complex, we show that addition of recombinant p21 disrupts the interaction between cdk2 and the E2F-p130 complex. To obtain in vivo evidence for this disruption, we used a cell line that expresses a temperature-sensitive p53. p21 is a p53-induced protein (15, 18). Activation of p53 in this cell line causes a time-dependent accumulation of p21. Analysis of the extracts of this cell line indicates a strong correlation between the levels of p21 and the interaction of cdk2 with the E2F-p130 complex. We also provide evidence that expression of p21 causes a specific repression of E2F-dependent transcription.

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MATERIALS AND METHODS

Assay of the E2F complexes. The E2F complexes were assayed by using a previously published procedure (58), with slight modification. The extracts or the column fractions were incubated in reaction mixtures (30-μl total volume) containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 40 to 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 20 μg of salmon sperm DNA per ml, 0.01% Nonidet P-40, and 0.2 ng of ^{32}P-labeled DNA probe. The DNA probe corresponded to a 100-bp fragment containing the adenovirus E2 promoter in which the upstream E2F site was mutated (58). Thus, the probe contained a single E2F-binding site. The ATF site was not functional under the assay conditions used. After the reaction mixtures were incubated for 20 to 30 min at room temperature, 4 μl of 20% Ficoll was added. Aliquots (7.5 μl) of the reaction mixtures were analyzed in gel retardation assays as described before (58). Electrophoresis was carried out in the presence of 0.5× Tris-borate-EDTA.

Fractionation of L-cell extracts for the E2F-p130-cyclin-cdk2 complex. Fractionations of L-cell extracts through heparin-agarose and Q-Sepharose columns were carried out essentially as described by Arroyo et al. (1) for purification of the E2F-cyclin A complex. Briefly, the 0.6 M KCI eluate from the heparin-agarose column contained the E2F complexes. This heparin-agarose material was dialyzed for 3 h against the Q-Sepharose equilibration buffer. The dialyzed material was fractionated by Q-Sepharose chromatography as described before (1).

Cell lines and tissue culture. Spinner cultures of mouse L cells were grown as described before (1). NIH 3T3 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. The PC-3 cell line and the status of p53 in this cell line are described elsewhere (37). The cell line expressing the temperature-sensitive mutant of p53 was constructed by Kokontis et al. (40).

Antibodies. Anti-p107 monoclonal antibody SD15 was a kind gift of N. Dyson and E. Harlow. Antisera against cyclin A were gifts from T. Hunter (Salk Institute) and J. R. Nevins (Duke University Medical Center). The p130 antibody was obtained from Santa Cruz Biotechnology. Each batch of the p130 antibody was titrated for reduction of cross-reaction with p107. Antibodies against cdk2 and cdk4 were purchased from Upstate Biotechnology.

Immunoprecipitation and Western blotting (immunoblotting). Immunoprecipitations were performed as described by Harlow and Lane (25). Western blotting was performed as described before (51), with the following modifications. After incubation with the primary antibody, the blots were washed and probed with a secondary antibody linked to horseradish peroxidase (Amersham Co.). The blots were developed with ECL (enhanced chemiluminescence) reagents as instructed by the manufacturer (Amersham Co.).

GST-p21 and plasmid CMV-p21. Plasmid CMV-p21 was a gift from Stephen Elledge, Baylor College of Medicine. To obtain glutathione *S*-transferase (GST)–p21, a full-length cDNA fragment corresponding to p21 was cloned into pGEX-2T in frame with GST. The fusion protein was purified from bacterial culture induced with isopropylthiogalactopyranoside (IPTG), using a procedure described for GST-E7 purification (1).

Transfections and CAT assays. Transfections and chloramphenicol acetyltransferase (CAT) enzyme assays were carried out as previously described (51).

RESULTS

Fractionation of L-cell extracts and partial purification of a cdk2-containing E2F-p130 complex. We have described isolation of the E2F-p107-cyclin A-cdk2 complex from mouse L-cell extracts. The purification steps involved chromatographies through a heparin-agarose, a Q-Sepharose, a phosphocellulose, and a DNA affinity column (1). The most highly purified preparation contained p107 as the major retinoblastoma family protein, as judged by silver staining and recognition by a p107-specific antibody (1). At the earlier stages of purification, however, a p130-containing E2F complex copurified with the E2F-p107-cyclin A-cdk2 complex. A gel retardation assay and a Western blot of the Q-Sepharose column fractions containing the E2F complexes are shown in Fig. 1. The fractions that contained the major DNA-binding complex of E2F also contained p107, p130, cyclin A, and cdk2. The p130 antibody (Santa Cruz) cross-reacted with p107. This antibody was used at a high dilution (1:500 to 1:1,000) to reduce the cross-reaction with p107. Besides copurification, we also observed that p130 in these fractions was associated with cdk2. The immunoprecipitates obtained by the cdk2 antibody also contained p130 (Fig. 1B, right panel).

To investigate whether p130 in the Q-Sepharose fractions was bound to E2F, we added antibodies to the gel shift reaction mixtures. Addition of saturating amounts of a monoclonal

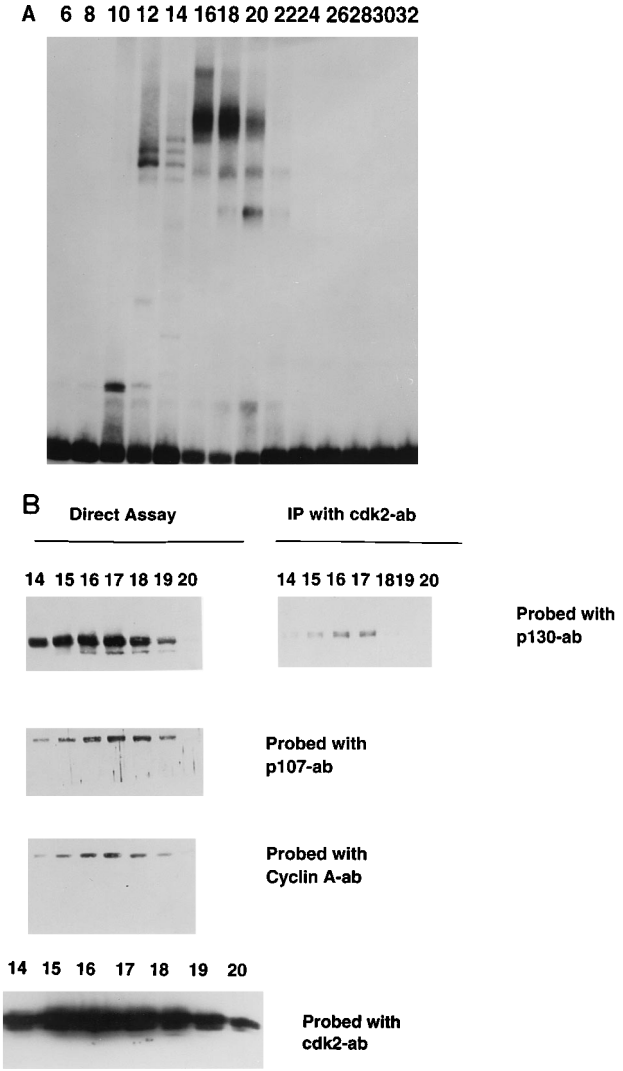

FIG. 1. Copurification of p130 with cdk2 and E2F-complexes (A) Mouse L-cell extracts were fractionated through heparin-agarose and Q-Sepharose columns as described in Materials and Methods. The indicated gradient fractions obtained after the Q-Sepharose chromatography were subjected to an E2F-specific DNA binding assay as described in Materials and Methods. (B) The indicated Q-Sepharose column fractions (50 μl) were subjected to Western blot analyses. The Western blots were probed with a p130 antibody (P130-ab; 1:500, final dilution), p107 antibody (1:100, final dilution), cyclin A antibody (1:200, final dilution), and cdk2 antibody (1:200, final dilution). The blots were developed by ECL. For, the p130 blot (right panel), the column fractions (100 μl) were immunoprecipitated (IP) with the cdk2 antibody. The immunoprecipitates were analyzed in Western blot assays for the presence of p130. The faster-migrating band in the p130 blot (left panel) has a molecular weight similar to that of p107.

antibody against p107 (SD15) did not alter the mobility of band M in a quantitative way; only a part of the band was recognized by the antibody (Fig. 2A). Similarly, when a saturating amount of a p130-specific antibody was used, only a part of band M was recognized (Fig. 2A). However, when a cocktail of p107 and p130 antibodies was used, band M was quantitatively supershifted. This result suggested that band M contained a mixture of two complexes: one contained p107 and the other contained p130. The p130 antibody (Santa Cruz) cross-reacted with the p107 complex only when used at a high concentration (1:30, final dilution). At lower concentration (1:150), we did not detect any cross-reaction with the p107-containing complex (not shown).

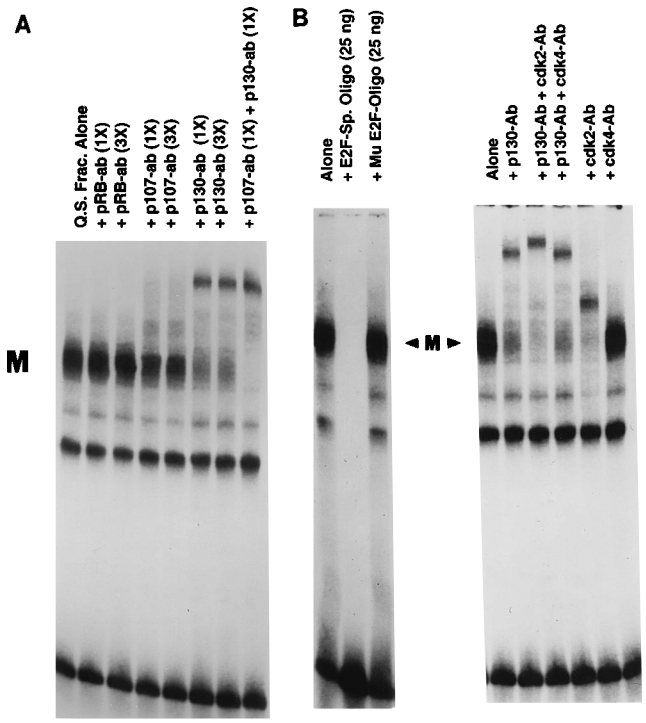

FIG. 2. p130 and cdk2 are in the same complex with E2F. (A) Q-Sepharose fraction (Q. S. Frac.) 19 (1 μg) was incubated in reaction mixtures that contained components necessary for the sequence-specific DNA binding of E2F. After incubation for 20 min at room temperature, p107 antibody (p107-ab) SD15 (1:270 or 1:90, final dilution) or a p130 antibody (1:450 or 1:150, final dilution; Santa Cruz) was added to the reaction mixtures. The incubation was continued for an additional 40 min at room temperature, and aliquots of the reaction mixtures were analyzed by gel retardation assays. Sp., specific; Oligo, oligonucleotide. (B) One microgram of Q-Sepharose fraction 19 was subjected to an E2F-specific gel retardation assay (in a 30-μl total reaction volume; see Materials and Methods) in the presence of competitor DNA and specific antibodies. For the antibody experiments, 0.1 μg of the cdk2 or cdk4 antibody, 0.01 μg of the p130 antibody, or a combination of the indicated amounts was added after a 20-min incubation for DNA binding. The p130 antibody does not recognize native p107 at this dilution (also see reference 67). The faster-migrating bands in the gel have not been characterized. Mu, mutant.

Hannon et al. (24) and Li et al. (47) provided evidence for binding of cyclins and cdk2 to p130. Li et al. provided evidence for an endogenous association of cyclin A and cyclin E with p130 (47). Moreover, a cyclin A-cdk2 complex has been shown by Cobrinik et al. (11) to interact with the E2F-p130 complex. Since the Q-Sepharose fractions described above contained p130 in complex with cdk2 (Fig. 1B), we investigated whether these proteins were in the same complex with E2F. Addition of the p130 antibody (1:450, final dilution) supershifted part of the M complexes, whereas an addition of the cdk2 antibody supershifted the majority of the M complexes (Fig. 2B). When p130 and cdk2 antibodies were added together, the p130 antibody-specific complex disappeared and a new, more slowly migrating complex was observed (Fig. 2B). This new complex was not observed when the cdk4 antibody was added instead of the cdk2 antibody. These results suggested that cdk2 and p130 were in the same complex with E2F.

p21 disrupts the interaction between cdk2 and the E2F-p130 complex. To investigate the effect of p53 on the cell cycle-regulated complexes of E2F, we analyzed the effect of p21 on the cdk2-containing complexes of E2F. p21 is a p53-induced inhibitor of the cell cycle (15, 18). It has been shown to be a universal inhibitor of the cdks (22, 26, 54, 69, 72). To determine the effects of p21 on the cdk2-containing E2F-p130 complex, a GST-p21 fusion protein was added to reaction mixtures that contained the Q-Sepharose purified material described above and components necessary for the E2F-specific DNA binding. Addition of GST-p21 resulted in a complete loss of the M complex (Fig. 3A and B). The loss of the M complex was accompanied by the appearance of three new bands, 1, 2, and 3 (band 3 comigrated with an uncharacterized band 3*). These faster-migrating complexes most likely are products of a p21-mediated disruption of complex M. Specific antibodies were used to characterize the newly generated bands. None of these faster-migrating bands was recognized by the cdk2 antibody (Fig. 3A, lane 8). However, addition of the p130 antibody supershifted bands 1 and 2 (Fig. 3B). It is unclear why we detected two p130-specific complexes (bands 1 and 2). It is possible that one of them contained modified or protease-digested components. A p107-specific antibody supershifted band 3, which is consistent with the fact that complex M contained a cdk2-containing E2F-p107 complex. The Q-Sepharose fraction was free of the E2F-pRB complex (not shown), and a pRB-specific antibody had no effect on the faster-migrating complexes.

Interestingly, band 1, which was generated by the addition of GST-p21, was similar in mobility to the G_0-specific E2F-p130 complex found in the extracts of serum-starved NIH 3T3 cells (Fig. 3A, lane 10). A comparison between the E2F complexes found in serum-starved NIH 3T3 cell extracts and those in serum-stimulated extracts is shown in Fig. 3C. Consistent with previously published results (11), the p130-containing E2F complex also contained cdk2 in the extracts from the serum-stimulated cells (the p130 antibody-specific complex was also recognized by the cdk2 antibody; compare lanes 5, 7, and 8 in Fig. 3C), whereas the p130-containing E2F complex lacked cdk2 in extracts from the serum-starved cells (compare lanes 2 and 4 in Fig. 3C). Thus, band 1 and the G_0-specific complex both contained E2F and p130 and had similar mobilities in gel retardation assays. Also, they were not recognized by the cdk2 antibody. A likely explanation of the results presented above is that p21 dissociates the cyclin-cdk2-containing complex to generate E2F complexes lacking cdk2 and that one of these complexes resembles the G_0-specific E2F-p130 complex.

The dissociation is independent of the DNA-binding activity of the E2F complexes and can be detected by coimmunoprecipitation assays. For example, addition of GST-p21 to the Q-Sepharose preparation (described above) resulted in binding of GST-p21 to cdk2, which was detected by coprecipitation of p21 via cdk2 by a cdk2 antibody (Fig. 3D, upper panel). The binding of p21 coincided with a loss of interaction between cdk2 and p130. We could easily detect coprecipitation of cdk2 with p130 when the Q-Sepharose preparation was subjected immunoprecipitation with the p130 antibody (Fig. 3D, lower panel). However, this coprecipitation of cdk2 became undetectable when the immunoprecipitation was carried out after preincubation of the Q-Sepharose material with GST-p21. This loss of interaction between cdk2 and p130 in the presence of p21 clearly confirms the results obtained by gel retardation assays.

p53-induced expression of p21 correlates with a disruption of the interaction between cdk2 and the E2F-p130 complex. To obtain evidence for an in vivo dissociation, we used a human prostate carcinoma cell line (PC-3) that expresses a temperature-sensitive mutant of human p53. Both human and mouse cells contain the polypeptides found in the E2F-p130-cyclin-cdk2 complex. Moreover, in the extracts of PC-3 cells, we could detect an E2F complex that is indistinguishable from the mouse E2F-p130-cyclin-cdk2 complex (not shown; see Fig. 5).

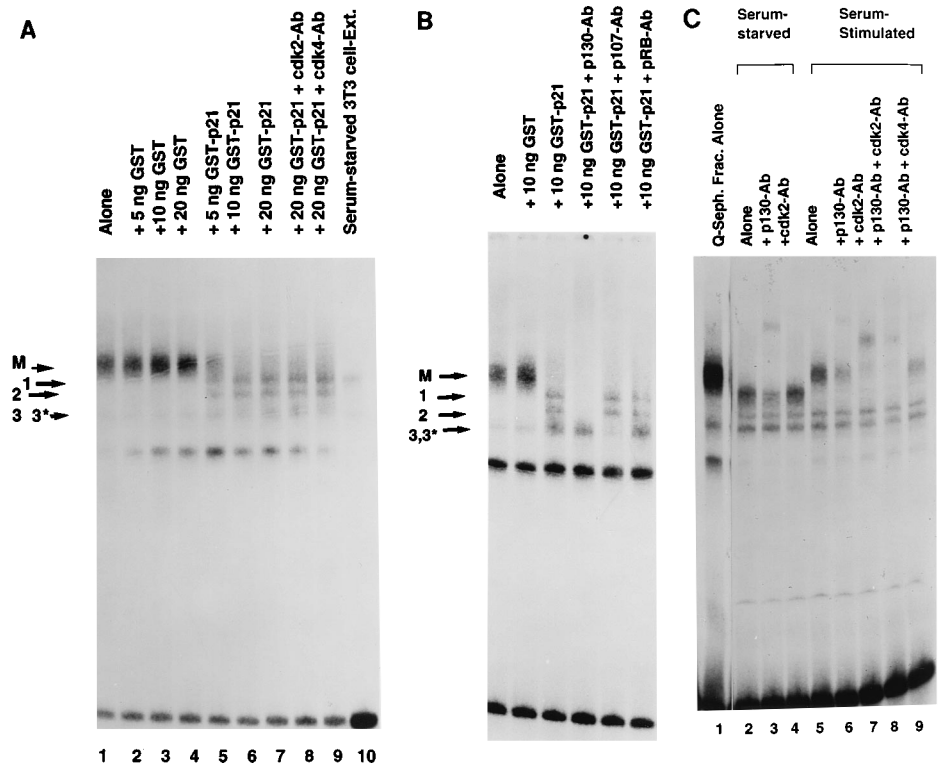

FIG. 3. GST-p21, in vitro, disrupts the interaction between cdk2 and the E2F-p130 complex. (A) One microgram (3 μl) of Q-Sepharose fraction 19 was incubated with the indicated amounts of GST or GST-p21 fusion protein in reaction mixtures that contained components necessary for the E2F-specific DNA binding (see Materials and Methods). Aliquots of the assay mixtures were analyzed by gel retardation assay. The reaction mixtures in lanes 8 and 9 also contained 0.1 μg of cdk2 and cdk4 antibodies (cdk2-Ab and cdk4-Ab), respectively. The reaction mixture in lane 10 contained 10 μg of extracts from serum-starved NIH 3T3 cells (cell-Ext.) instead of the Q-Sepharose fraction. (B) Q-Sepharose fraction 19 was incubated in an E2F-specific DNA-binding reaction mixture in the presence or absence of GST (10 ng) or GST-p21 (10 ng) protein. After incubation for 30 min at room temperature, the indicated antibodies (1:300, final dilution) were added to the reaction mixtures. The p107 antibody was a monoclonal antibody (SD15; gift of Ed Harlow's laboratory). The pRB antibody was a monoclonal antibody from Pharmingen. (C) NIH 3T3 cells at 80% confluence were incubated in serum-free medium for 36 h. The cells were then stimulated by changing the medium to serum (10% fetal bovine serum)-containing medium. Extracts were prepared from cells harvested at various times after stimulation. A gel retardation assay for E2F in the serum-starved extracts and in the extracts of 14-h serum-stimulated cells is shown. For the antibody experiments, the cdk2 (0.1 μg), cdk4 (0.1 μg), or p130 (0.01 μg) antibody or a combination of the indicated amounts was added to the assay mixtures. Lane 1 shows the mobility of the M complex. Q-Seph. Frac., Q-Sepharose fraction. (D) Q-Sepharose fraction 19 (10 μg) was incubated either alone or in the presence of 0.1, 0.5, 1.0, 2.0, 5.0, or 10 ng of GST-p21. After incubation for 20 min at room temperature, the reaction mixtures were subjected to immunoprecipitation (IP) with the cdk2 or p130 antibody. The immunoprecipitates were further analyzed by Western blotting. In the upper panel, the immunoprecipitates obtained with the cdk2 antibody were analyzed for the presence p21; a p21-specific antibody was used to probe the blot. In the lower panel, the immunoprecipitates obtained with the p130 antibody were analyzed for the presence of cdk2; a cdk2-specific antibody was used to probe the blot. The blots were developed by ECL as described in Materials and Methods.

This p53-expressing cell line was constructed by introducing a temperature-sensitive mutant of p53 mutated at position 138 from alanine to valine into $p53^{-/-}$ PC-3 prostate carcinoma cells (40). When grown at 39°C, these cells express an inactive p53. The mutant p53 protein is active at 32°C. Consequently, a temperature shift to 32°C results in growth arrest (40). p21 has been shown to be transcriptionally induced by p53 (15, 18). We assayed for p21 in extracts of these cells after incubation at 32°C for various time periods. The cells growing at 39°C contained very little p21 (Fig. 4A, lane 0h). When the incubation temperature was decreased to 32°C, there was a time-dependent accumulation of the p21 protein. The levels of several other proteins such as cdk2 and DP1 (a subunit of E2F [21, 35]) were unaltered during this temperature shift (Fig. 4A). If p21 disrupts the interaction between cdk2 and the E2F-p130 complex, we would not expect to find the interaction in extracts that contain higher levels of p21. The interaction between cdk2 and p130 or E2F was analyzed by coimmunoprecipitation. The immunoprecipitates obtained with a p130 or DP1 antibody were probed with a cdk2 antibody in Western blot assays. cdk2 protein was coprecipitated with p130 as well as DP1 from

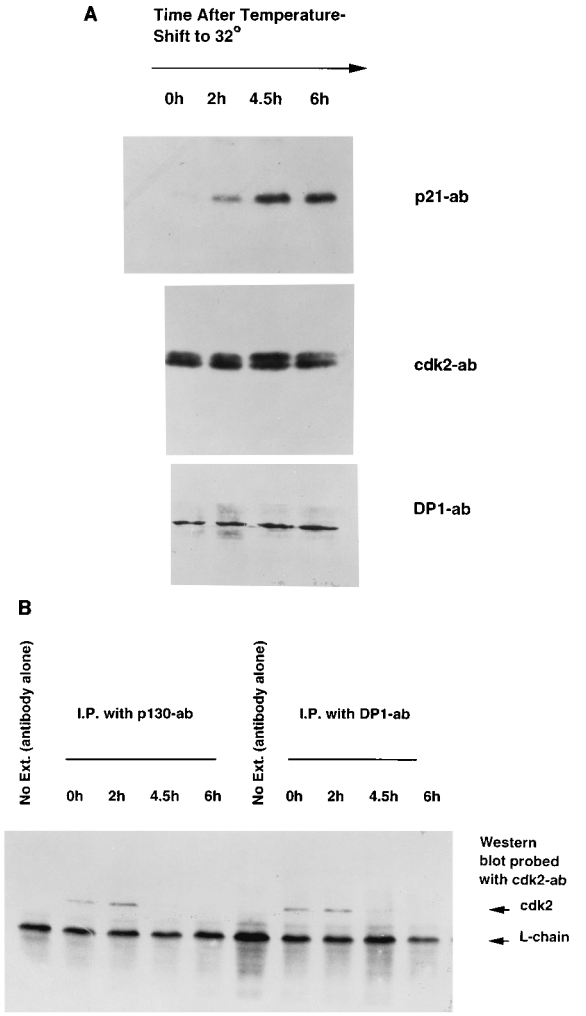

FIG. 4. p53-induced expression of p21 correlates with a loss of interaction between cdk2 and p130 or DP1. PC-3 cells were incubated at 32°C (a temperature at which the mutant p53 is functionally active) for the indicated periods of time. The cell extracts were analyzed for p21, cdk2, and DP1 by Western blot assays (A). The cell extracts (150 μg of protein) were also subjected to immunoprecipitation (I.P.) experiments using a p130 antibody or DP1 monoclonal antibody (gift of Chin-Lee Wu, Ed Harlow's laboratory) (p130-ab or DP1-ab). The immunoprecipitates were analyzed in Western blot assays for cdk2 (B). The Western blots were developed by ECL.

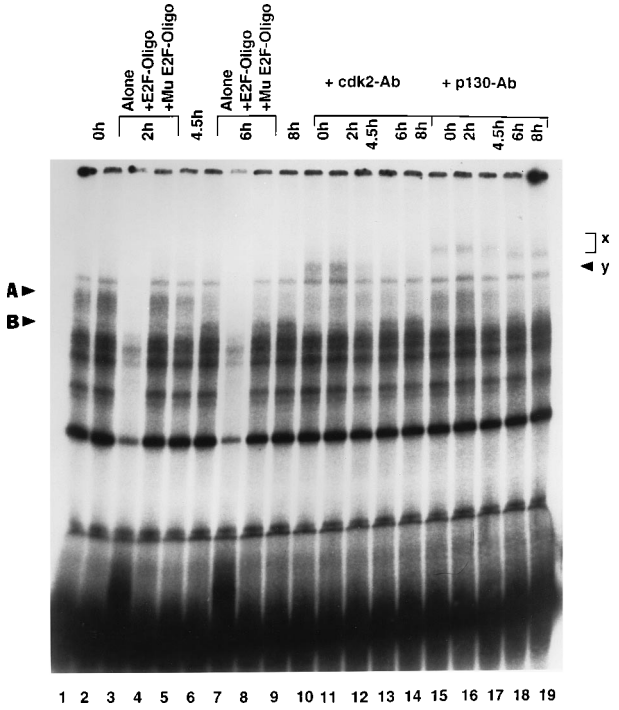

FIG. 5. p53-induced expression of p21 correlates with a disruption of the interaction between cdk2 and the E2F-p130 complex. The extracts (7.5 μg) obtained after p21 induction (as in Fig. 4) were subjected to gel retardation assays for the E2F complexes. An E2F-specific oligonucleotide (E2F-Oligo; 25 ng) or a mutant (Mu) oligonucleotide was used as a competitor to identify the E2F-specific complexes in the extracts (2- and 6-h time points). For experiments in lanes 10 to 14, the reaction mixtures also contained 0.1 μg of the cdk2 antibody (cdk-Ab); for experiments in lanes 15 to 19, the reaction mixtures contained 0.01 μg of the p130-antibody (p130-Ab). Band A was detected only in extracts corresponding to 0- and 2-h induction for p21; band B was observed in extracts corresponding to 4.5-, 6-, and 8-h induction for p21. Bands marked x were obtained in the presence of the p130 antibody, and band y was obtained in the presence of the cdk2 antibody.

extracts that contained lower levels of p21 (Fig. 4B, lanes 0h and 2h). However, extracts that contained higher levels of p21 showed very little coprecipitation of cdk2 with either p130 or DP1 (Fig. 4B, lanes 4.5h and 6h). This loss of interaction between cdk2 and p130 or DP1 was not a consequence of cell cycle arrest, because these cells did not arrest after 6 h incubation at the permissive temperature (data not shown and reference 40). A temperature shift of the parental $p53^{-/-}$ to 32°C for 6 h cell line had no effect on the interaction between cdk2 and p130 (not shown). Thus, an increase in the levels of p21 correlates with a loss of interaction between cdk2 and p130 or E2F.

The extracts containing various levels of p21 were also analyzed by gel retardation assays. These extracts contained multiple E2F-specific complexes. Two of these bands (A and B in Fig. 5) are relevant to this study. The other E2F-specific bands in these extracts remain to be characterized. Band A (which was similar in mobility as to band M; data not shown) was observed in extracts containing lower levels of p21 (0- and 2-h time points), and the extracts with higher levels of p21 exhibited a marked reduction of the intensity of this band (compare lane 1 or 2 with lane 6 or 9). The reduction of band A was accompanied by an increase in the intensity of a faster-migrating band B (compare lane 1 or 2 with lane 6 or 9; unfortunately, band B is partly masked by other bands). This apparent conversion of band A to band B is consistent with a loss of cdk2 from band A. Band A contained cdk2, as it was supershifted by the cdk2 antibody; band B was not recognized by the cdk2 antibody. Moreover, the cdk2 antibody-specific supershift (band Y in Fig. 5) was seen only in extracts with lower levels of p21 (compare lanes 10 to 14 in Fig. 5). Further evidence for the dissociation of the cdk2-containing E2F-p130 complex was observed when a p130 antibody was added to reaction mixtures. The p130 antibody-specific complex migrated faster in extracts that contained higher levels of p21, indicating a loss of components from the p130-containing complex (Fig. 5; compare lanes 15 to 19 for the bands marked x). Bands A and B corresponded to a mixture of p107- and p130-containing complex (not shown). We detected only a partial effect by adding the p130 antibody, because the experiment was designed to assay specifically the p130-containing complex. Taken together, these results clearly support the in vitro observation that p21

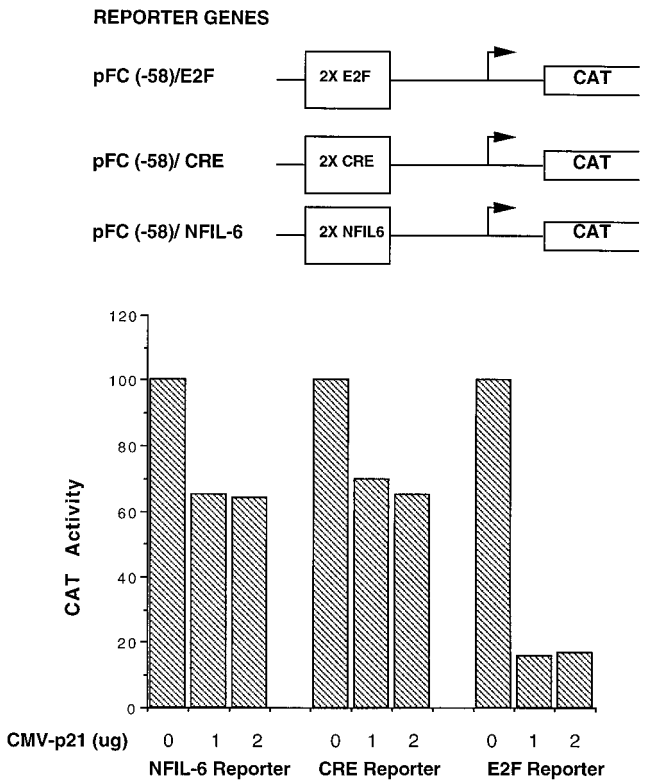

FIG. 6. Expression of p21 reduces E2F-dependent transcription. The reporter CAT gene constructs contained human c-*fos* gene sequences between −58 and +42 upstream of the CAT coding sequences. The constructs also contained oligonucleotides corresponding to two copies of the E2F-, cAMP response element (CRE)-, or NFIL-6-binding site as shown in the diagram. Three micrograms of the reporter construct was transfected into NIH 3T3 cells along with the indicated amounts of plasmid expressing p21. Plasmid CMV-βgal was used as a transfection control. Transfections and CAT activity assays were performed as previously described (2, 51). Levels of conversion of chloramphenicol were determined for each transfection. These data were then corrected for variabilities in transfection efficiencies by analysis of β-galactosidase expression. Conversion levels for each promoter construct without the presence of p21 were normalized to 100%.

disrupts the interaction between cdk2 and the E2F-p130 complex.

Expression of p21 reduces E2F-dependent transcription. p21 not only disrupts the binding of cdk2 with the E2F-p130 complex but also disrupts the binding of cdk2 with the E2F-p107 complex (Fig. 3B and reference 74). To analyze the consequences of this disruption, we determined the effects of p21 expression on E2F-dependent transcription. PC-3 cells, which were used to demonstrate a p53-dependent disruption of the interaction between cdk2 and the E2F-p130 complex, contained a variety of other E2F complexes that were not affected by p21. NIH 3T3 cells, on the other hand, contain mainly cyclin-cdk2-containing complexes, which would be altered by p21. Transient transfections experiments were performed with NIH 3T3 cells to investigate the effects of p21 expression on E2F-dependent transcription. Three reporter CAT gene constructs were used; they all contained a basal c-*fos* gene promoter (sequences between −53 and +42). The only difference between these reporter constructs was in the upstream elements, as shown schematically in Fig. 6. To correct for the variabilities in transfection efficiencies, a plasmid (CMV-βgal) that expressed β-galactosidase was cotransfected. The results of the transfection experiments are summarized in Fig. 6. Expression of p21 reduced E2F-dependent transcription by five to sixfold, whereas CAT gene expression from the cyclic AMP (cAMP) response elements or the NFIL-6 reporter was only marginally affected by p21 expression. This result, by itself, does not prove but is consistent with the notion that p21 represses E2F-dependent transcription by disrupting the cdk2-containing complexes.

DISCUSSION

The mechanism by which p21 disrupts the interaction between cdk2 and the E2F-p130 complex is unclear. It is possible that p21 interacts with the same site within cyclin-cdk2 that is involved in the interaction with the E2F-p130 complex, and thus, in the presence of excess p21, the complex is dissociated by competition. The cyclin-cdk2-binding site within p130 is not known. However, p107, which is structurally related to p130, has been studied in great detail in this regard. Zhu et al. (74) identified a region in p107 (between residues 651 and 669) that is essential for binding to cyclin-cdk2. Interestingly, these authors also show that this region in p107 possesses homology to the N-terminal region (between residues 16 and 28) of p21. Moreover, the p21 region between residues 15 and 20 is important for binding to cyclin/cdk2 (74). Thus, it is likely that p21 would compete with p107 for cyclin-cdk2. p130 possesses extensive sequence homology with p107. Sequences similar to the p107's cyclin-cdk2 binding site can also be detected in p130 (between amino acid residues 672 and 688 [24, 47]). Interestingly, this region of p130 exhibits 47% sequence identity with the p21 region between residues 11 and 28 (not shown). Thus, we think that p21 disrupts the interaction between cdk2 and the E2F-p130 complex via a mechanism that is also involved in the disruption of the complex involving p107.

The significance of cdk2 binding to the E2F-p130 complex is not obvious. For the p107 complex, Zhu et al. (74) have shown that the cdk2 binding leads to a dissociation of the E2F-p107 complex resulting in a release of free E2F, which retains the DNA-binding function (74). Thus, the four-member complex might be an intermediate that eventually results in activation of E2F. In this scenario, cdk2 binding to the E2F-p130 complex might play an analogous role that eventually leads to activation of E2F. This view is consistent with the fact that the cdk2-bound complex is seen only in dividing cells. A p21-mediated disruption of the cdk2-bound complex would prevent activation of E2F.

The p21-mediated disruption is significant, because one of the products of the disruption is a complex that resembles the E2F-p130 complex found in growth-arrested cells. This two-member complex is believed to be involved in G_1 arrest and exit from the cell cycle (11, 67). The predominant E2F complex found in terminally differentiated muscle cells is that of E2F and p130 (62). In addition, p130 has also been shown to be a downstream target of the growth-inhibitory function of transforming growth factor-β1 (30). Treatment of cells with transforming growth factor-β1 results in an accumulation of the E2F-p130 complex (30). Thus, we think that an accumulation of the E2F-p130 complex (as well as the E2F-p107 complex) plays a role in the cell cycle-inhibitory function of p21.

The observations described here may have implications for the growth-inhibitory function of the tumor suppressor p53. Using a cell line that expresses a temperature-sensitive mutant of p53, we have shown that activation of p53 results in a disruption of the interaction between cdk2 and the E2F-p130 complex. The disruption coincided with an accumulation of p21. Our interpretation is that p53, by inducing p21 synthesis, causes a disruption of the interaction between cdk2 and the

E2F-p130 complex. This view is consistent with the in vitro data for recombinant p21. Additionally, in transient transfection assays, expression of p21 specifically reduces E2F-dependent transcription (Fig. 6). On the basis of these results, we speculate that an increase in the level of p21 would raise the level of the G_0/G_1 form of the E2F-p130 complex, which in turn would repress certain E2F-dependent genes. p21 is believed to be one of the mediators of p53-induced cell cycle arrest (15, 18). Results presented here, therefore, link the p53 suppression pathway to the cell cycle-regulatory complexes of E2F.

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