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Cyclin A/CDK2 Binds Directly to E2F-1 and Inhibits the DNA-Binding Activity of E2F-1/DP-1 by Phosphorylation

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E2F-1, a member of the E2F transcription factor family, contributes to the regulation of the G_1-to-S phase transition in higher eukaryotic cells. E2F-1 forms a heterodimer with DP-1 and binds to several cell cycle regulatory proteins, including the retinoblastoma family (RB, p107, p130) and cyclin A/CDK2 complexes. We have analyzed E2F-1 phosphorylation and its interaction with cyclin A/CDK2 complexes both in vivo and in vitro. In vitro, E2F-1 formed a stable complex with cyclin A/CDK2 but not with either subunit alone. DP-1 did not interact with cyclin A, CDK2, or the cyclin A/CDK2 complex. While the complex of cyclin A/CDK2 was required for stable complex formation with E2F-1, the kinase-active form of CDK2 was not required. However, E2F-1 was phosphorylated by cyclin A/CDK2 in vitro and was phosphorylated in vivo in HeLa cells. Two-dimensional tryptic phosphopeptide mapping studies demonstrated an overlap in the phosphopeptides derived from E2F-1 labeled in vitro and in vivo, indicating that cyclin A/CDK2 may be responsible for the majority of E2F-1 phosphorylation in vivo. Furthermore, an active DNA-binding complex could be reconstituted from purified E2F-1/DP-1 and cyclin A/CDK2. Binding studies conducted both in vitro and in vivo demonstrated that the cyclin A/CDK2-binding region resided within the N-terminal 124 amino acids of E2F-1. Because the stable association of E2F-1 with cyclin A/CDK2 in vitro and in vivo did not require a DP-1- or RB-binding domain and because the interactions could be reconstituted from purified components in vitro, we conclude that the interactions between cyclin A/CDK2 and E2F-1 are direct. Finally, we report that the DNA-binding activity of the E2F-1/DP-1 complex is inhibited following phosphorylation by cyclin A/CDK2.

E2F was originally discovered by Kovesdi et al. (38) as a target of the adenovirus E1A oncogene. Characterization and purification of E2F established its binding-site specificity and several of its biochemical properties (63, 64). Later work demonstrated that E2F was involved in the regulation of several genes and proto-oncogenes whose activities control cell growth and/or the cell cycle events. Examples include c-*myc*, c-*myb*, *DHFR*, the retinoblastoma gene, and *cdc2* (7, 16, 29, 40, 46, 48, 57, 60, 61). The involvement of E2F in the expression of these genes suggested that the activities of E2F would contribute to the regulation of cellular proliferation and of the cell division cycle (for reviews, see references 23, 41, and 49) Indeed, the expression of E2F-1 or microinjection of an E2F-1 cDNA promotes the entry of REF-52 cells into S phase (36).

The cDNA cloning of E2F-1 and associated family members has provided important tools for the dissection of regulatory pathways. To date, three human E2F species and a related protein, DP-1, have been reported by several groups (20, 25, 35, 37, 43, 45, 58). The E2F family members share extensive homology and contain a DNA-binding domain in the N-terminal portion that is characterized by a basic region and a motif with some homology to a helix-loop-helix structure. The transcriptional activation function was localized to a C-terminal region, and the binding site for growth suppressor proteins is nested within this region (15, 25, 37). Growth suppressors such as the retinoblastoma gene product (RB) and p107 have been demonstrated to inhibit E2F-dependent transcription (3, 6, 9, 11, 27, 31, 53, 54, 56, 65, 67). Another RB-related protein (p130) also interacts with E2F (13, 22, 44). The intermediate region adjacent to the DNA-binding site contains a leucine zipper-like motif and is the site of heterodimeric interactions of E2F-1 and DP-1. The salient features of the DP-1/E2F-1 protein-protein interaction are high-affinity binding to E2F sites and efficient transcriptional activation (4, 26, 39). Therefore, heterodimeric protein-protein interactions between E2F and DP family members and a growing list of regulatory proteins contribute to combinatorial regulation of E2F transcriptional function.

A direct link between E2F and cell cycle regulation was suggested by the discovery that E2F exists in distinct complexes with cyclin E/CDK2 and cyclin A/CDK2 in the G_1 and S phases, respectively, of the cell cycle. The presence of an active CDK2 kinase in an E2F complex is intriguing in the context of earlier work which indicated that phosphorylation contributes to the regulation of E2F function (2). Complexes containing E2F and cyclinA/CDK2 in vivo also reportedly contain p107 (5, 8, 12, 18, 42, 50, 59). In contrast to RB, p107 contains distinct binding sites for cyclin A (19), but both growth suppressors contain binding sites for E2F. It has been proposed that the role of p107 is the targeting of cyclin A/CDK2 to E2F complexes (18, 59).

In this report, we examine the interactions between E2F-1, DP-1, cyclin A, and CDK2. E2F-1 formed a stable complex with cyclin A/CDK2 complexes in vitro but not with either subunit alone. The cyclin A/CDK2-binding region mapped to the N-terminal 124 amino acids of E2F-1, and this region was sufficient for E2F-1 binding to cyclin A/CDK2 in vivo. Because

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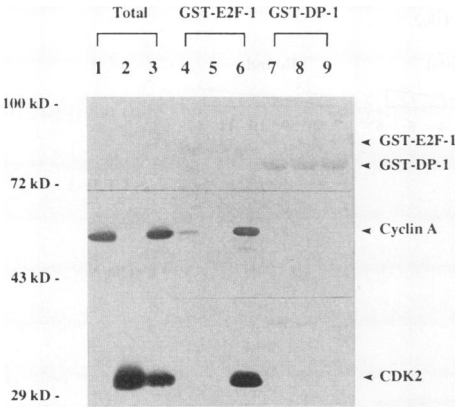

FIG. 1. Cyclin A/CDK2 interacts directly with E2F-1 but not with DP-1 in vitro. Lysates were prepared from insect cells infected with recombinant viruses encoding either cyclin A (lanes 1, 4, and 7), CDK2 (lanes 2, 5, and 8), or cyclin A and CDK2 (lanes 3, 6, and 9). Lysates were normalized to 1 mg/ml by the addition of lysates prepared from insect cells infected with wild-type virus. Fifty micrograms of each lysate was resolved directly by SDS-PAGE on a 10% gel (lanes 1 to 3). Alternatively, 500 μg of each lysate was incubated with either 2 μg of GST–E2F-1 (lanes 4 to 6) or 3 μg of GST–DP-1 (lanes 7 to 9). Bound proteins were resolved by SDS-PAGE on a 10% gel and transferred to nitrocellulose. GST–E2F-1 and GST–DP-1 were visualized by staining the nitrocellulose with Ponceau S (top). Cyclin A and CDK2 were visualized by incubating the nitrocellulose with sera specific for either cyclin A (middle) or CDK2 (bottom).

the stable association of E2F-1 with cyclin A/CDK2 in vitro and in vivo required only the first 124 amino acids of E2F-1 and could be reconstituted from purified components in vitro, we conclude that the interaction of cyclin A/CDK2 with E2F-1 is direct. Furthermore, the interactions of cyclin A/CDK2 with E2F-1 define a new functional domain, since no protein-protein interactions have been described for this N-terminal region of E2F-1. Because different regions of the E2F-1 protein are utilized, the direct binding of E2F-1 to cyclin A/CDK2 is also independent of DP-1 and RB family members. Finally, we report that the DNA-binding activity of the E2F-1/DP-1 complex is inhibited following phosphorylation by cyclin A/CDK2.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum and 1 mM glutamine at 37°C in the presence of 5% CO_2. *Spodoptera frugiperda* Sf9 cells were cultured as described previously (52).

Generation of recombinant baculovirus. Recombinant viruses encoding human cyclin A, glutathione *S*-transferase (GST)–cyclin A, and CDK2 have been described previously (1, 50).

For generation of recombinant virus encoding $CDK2^-$ (N132A), pET-HACDK2(N132A) (14) was digested with *Nde*I, and an *Nde*I-*Bam*HI linker was inserted. $CDK2^-$(N132A) was excised as a *Bam*HI fragment and was inserted into the *Bam*HI site of pVL1393. Recombinant virus was generated as described previously (51).

Antibodies. C160 (a monoclonal antibody specific for human cyclin A) was kindly provided by Ed Harlow. Affinity-purified CDK2 antibody (made against a C-terminal peptide derived from human CDK2) was prepared by Brian Gabrielli. SQ11 (a monoclonal antibody specific for E2F-1) was kindly provided by Bill Kaelin.

Generation of polyclonal antibodies specific for E2F-1. The plasmid encoding GST–E2F-1 was transformed into *Escherichia coli* JM109. A single bacterial colony was used to inoculate a 200-ml culture that was incubated at 37°C overnight. The 200-ml culture was diluted into 2 liters of fresh LB (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 0.1 mg of ampicillin per ml. After incubation at 37°C for 1 h, the diluted culture (optical density at 595 nm of 0.6 to 0.8) was induced with 0.5 mM isopropylthiogalactopyranoside (IPTG) for 4 h at room temperature. Induced bacteria were harvested by centrifugation at 5,000 rpm for 10 min in a Beckman JA-14 rotor. Pelleted bacteria were resuspended in 20 ml of Tris–Nonidet P-40 (NP-40) lysis buffer (50 mM Tris [pH 7.4], 0.1% NP-40, 5 mM NaF, 10 mM $NaPP_i$, 250 mM NaCl), supplemented with 2 mM phenymethylsulfonyl fluoride (PMSF), 0.15 U of aprotinin per ml, 20 μM leupeptin, 20 μM pepstatin, 1 mg of lysozyme per ml. After rocking at 4°C for 20 min, pellets were sonicated three times for 10 s each with a probe tip sonicator at the microtip limit. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Pellets were resuspended in 10 ml of NETN (20 mM Tris [pH 8.0], 1 mM EDTA, 0.5% NP-40, 100 mM NaCl). Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer and were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel. Emulsified gel bands containing GST–E2F-1 were used to immunize two New Zealand White rabbits.

Preparation of affinity columns. Cultures of induced bacteria expressing GST (100 ml), GST–E2F-1 (400 ml), and GST-N76 (100 ml) were pelleted and lysed in 5 to 20 ml of Tris–NP-40 lysis buffer. Clarified lysates were incubated with 0.2 ml of packed glutathione-agarose beads per ml of lysate. Reactions were incubated at 4°C for 30 min. Precipitates were washed four times in NETN, and GST-containing proteins were eluted in 1 to 5 ml of NETN containing 20 mM glutathione at pH 7.0. Eluted proteins were dialyzed against morpholine propanesulfonic acid (MOPS) buffer (50 mM MOPS [pH 7.8], 500 mM NaCl) at 4°C overnight and were then adjusted to 8 ml by the addition of MOPS buffer. GST (0.75 mg/ml), GST–E2F-1 (0.45 mg/ml), and GST-N76 (0.1 mg/ml) were covalently coupled to 1 g of activated CH Sepharose 4B (Pharmacia) for 3 h at room temperature. Blocking and washing reactions were performed as instructed by the manufacturer.

Affinity purification of E2F-1 antibody. The GST–E2F-1 antisera were first passed over the GST column to remove antibodies specific for GST. The flowthrough fractions were then loaded onto a GST–E2F-1 column or a GST-N76 column. Columns were then washed with radioimmunoprecipitation assay buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA) and then with NETN buffer supplemented with 0.5 M NaCl. Antibodies specific for E2F-1 were eluted first with 100 mM glycine (pH 2.5) and then with 1% triethylamine (pH 11). All fractions were neutralized with 1 M Tris (pH 8.0) and then dialyzed against Tris-buffered saline (10 mM Tris [pH 8.0], 150 mM NaCl).

Generation of GST–E2F-1 deletion mutants. Deletion mutants of E2F-1 were generated from GST–E2F-1 (37, 58) as follows. To create N282, GST–E2F-1 was digested with *Bam*HI and *Bgl*II, and the 0.8-kb fragment encoding the N-terminal 282 amino acids of E2F-1 was inserted into *Bam*HI-linearized pGEX-2TK. To create C154, GST–E2F-1 was digested with *Bgl*II and *Eco*RI, and the 0.5-kb fragment encoding the C-terminal 154 amino acids of E2F-1 was inserted into *Bam*HI-

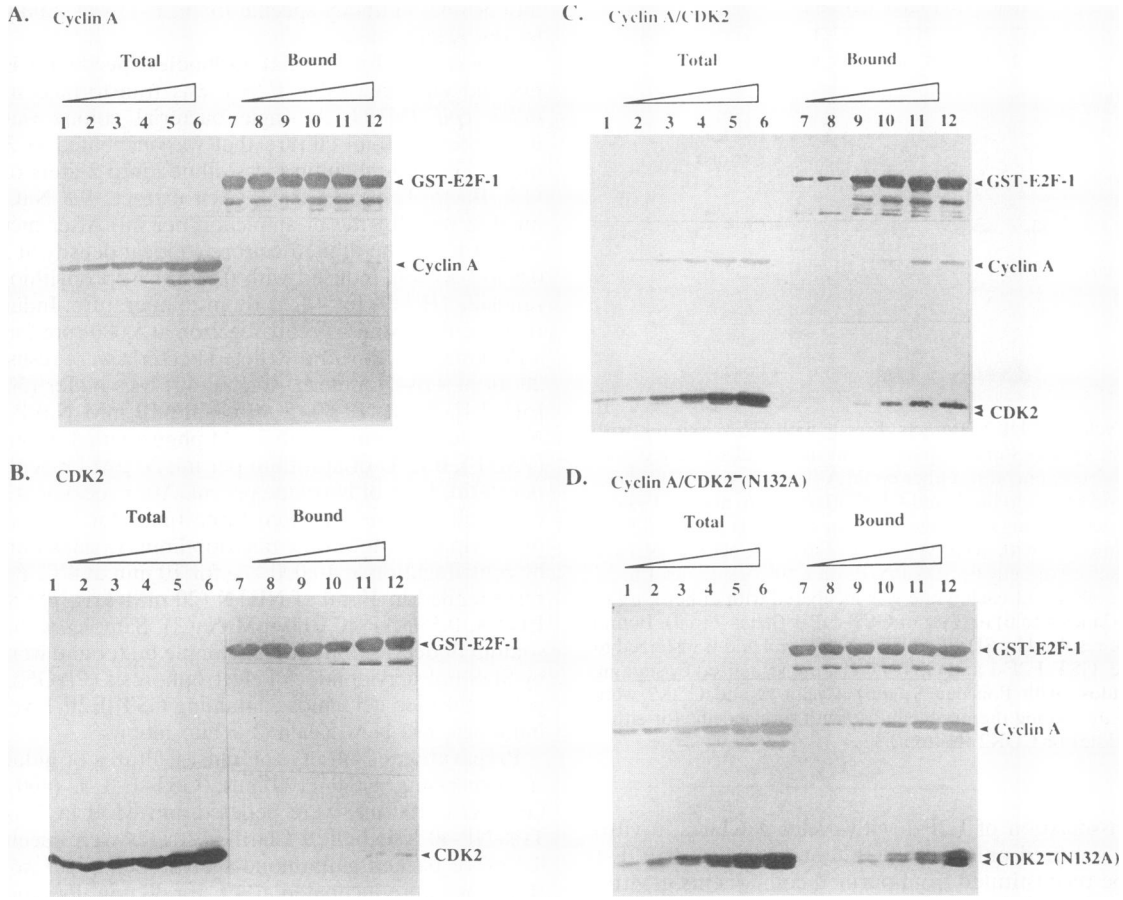

FIG. 2. Stable interaction between E2F-1 and cyclin A/CDK2 does not require kinase-active CDK2. Lysates were prepared from insect cells infected with recombinant viruses encoding cyclin A (A), CDK2 (B), cyclin A and CDK2 (C), or cyclin A and CDK2$^-$(N132A), a kinase-deficient mutant of CDK2 (D). Lysates were normalized to 0.4 mg/ml with lysates prepared from insect cells infected with wild-type virus. Differing amounts of each lysate were either resolved directly by SDS-PAGE (lanes 1 to 6) or incubated with 0.5 μg of GST–E2F-1 that had been affinity purified from *E. coli* by using glutathione-agarose (lanes 7 to 12). Proteins were resolved on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and probed with serum specific for either E2F-1 (SQ11; top), cyclin A (middle), or CDK2 (bottom). Amounts of lysate: 2.5 μg (lane 1); 5 μg (lane 2); 10 μg (lane 3); 20 μg (lane 4); 40 μg (lane 5); 80 μg (lane 6); 10 μg (lane 7); 20 μg (lane 8); 40 μg (lane 9); 80 μg (lane 10); 160 μg (lane 11); 320 μg (lane 12).

and *Eco*RI-digested pGEX-3X. To create N150, N282 was digested with *Sal*I and *Eco*RI, and the 5.4-kb fragment encoding the N-terminal 150 amino acids of E2F-1 was transformed directly into *E. coli* HB101. To create N76, N282 was digested with *Dra*III and *Eco*RI, and the 5.2-kb fragment encoding the N-terminal 76 amino acids of E2F-1 was transformed directly into *E. coli* HB101.

Production and purification of GST–DP-1, GST–E2F-1, and deletion mutants. *E. coli* JM109 cells were transformed with pGST-DP-1 (26) and the plasmids described above. GST–DP-1 and GST–E2F-1 expression was induced with 0.5 mM IPTG. Induced bacteria were harvested by centrifugation at 5,000 rpm for 10 min in a Beckman JA-14 rotor. Pelleted bacteria were resuspended in Tris–NP-40 lysis buffer supplemented with 2 mM PMSF, 0.15 U of aprotinin per ml, 20 μM leupeptin, 20 μM pepstatin, and 1 mg of lysozyme per ml (10 ml of induced bacterial culture per ml of Tris–NP-40 lysis buffer). After rocking at 4°C for 20 min, pellets were sonicated three times for 10 s each with a probe tip sonicator at the microtip limit. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Clarified lysates were incubated with glutathione-agarose beads (25 μl of packed beads per ml of lysate) for 30 min at 4°C. Beads were pelleted and washed with NETN, and in some cases, recombinant proteins were eluted with 20 mM glutathione (pH 7.0).

Cyclin A/CDK2 and E2F-1 interactions in vitro. Sf9 insect cells were infected with cyclin A virus or CDK2 virus or were coinfected with both cyclin A and CDK2 viruses [wild-type CDK2 or CDK2$^-$(N132A)]. Lysates were prepared from infected cells with NETN supplemented with 2 mM PMSF, 0.15 U of aprotinin per ml, 20 μM leupeptin, and 20 μM pepstatin at approximately 40 h after infection. Lysates were normalized to 1 mg/ml (Fig. 1) or 0.4 mg/ml (Fig. 2) by the addition of lysates prepared from insect cells infected with wild-type virus. Diluted lysates were then incubated for 1 h at 4°C with 2 to 5 μg of purified GST–E2F-1 or purified GST–DP-1 that was coupled to glutathione-agarose beads. The beads were pelleted and washed with NETN. Bound proteins were resolved on SDS–10% polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific for E2F-1 (SQ11), cyclin A (C160), and CDK2.

Phosphorylation of E2F-1 in vitro. (i) Purification of GST–E2F-1. Bacterial cultures (100 ml) induced for GST–E2F-1 expression were pelleted and resuspended in 4 ml of Tris–

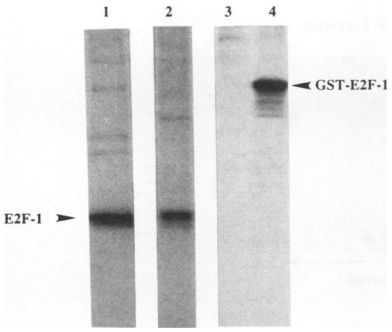

FIG. 3. Phosphorylation of E2F-1 in vitro by cyclin A/CDK2 and in vivo. Lane 1, HeLa cells were incubated with $^{32}P_i$. Lysates were prepared, and E2F-1 was immunoprecipitated with affinity-purified antibodies specific for full-length E2F-1. Immunoprecipitates were boiled in 1% SDS, and ^{32}P-labeled E2F-1 was reimmunoprecipitated with affinity-purified antibodies specific for E2F-1. Immunoprecipitates were resolved by SDS-PAGE on an 8% gel; ^{32}P-labeled E2F-1 was visualized by autoradiography. Lane 2, HeLa cells, transfected with a plasmid encoding E2F-1 under the control of the CMV promoter, were incubated with $^{32}P_i$. Lysates were prepared, and E2F-1 was immunoprecipitated with affinity-purified antibodies specific for full-length E2F-1. Immunoprecipitates were resolved by SDS-PAGE on an 8% gel; ^{32}P-labeled E2F-1 was visualized by autoradiography. Lanes 3 and 4, GST–E2F-1 was phosphorylated in vitro by purified monomeric CDK2 (lane 3) or GST-cyclin A/CDK2 (lane 4). Reaction products were resolved by SDS-PAGE on a 10% gel; ^{32}P-labeled GST–E2F-1 was visualized by autoradiography.

NP-40 lysis buffer supplemented with protease inhibitors as described above. Clarified lysates were incubated with 250 μl of packed glutathione-agarose beads at 4°C for 30 min. After being washed three times with Tris–NP-40 lysis buffer and twice with incomplete kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol), bound proteins were eluted in 500 μl of incomplete kinase buffer supplemented with 20 mM glutathione (pH 7.0). Fifty microliters of eluted protein was used for each kinase reaction.

(ii) Purification of CDK2 and GST-cyclin A/CDK2. Approximately 10^7 insect cells infected with either CDK2 alone or GST-cyclin A and CDK2 were lysed in 1 ml of coupling buffer (25 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4]), supplemented with various protease inhibitors as described above. Lysates were precleared by centrifugation at 10,000 × *g* for 10 min and incubated with 50 μl of packed ATP agarose beads (11 spacer; Sigma) at 4°C for 1 h. After three washes with coupling buffer, bound proteins were eluted with 50 μl of elution buffer (200 mM NaCl, 50 mM HEPES, 10 mM $MgCl_2$, 1 mM dithiothreitol [pH 7.4]).

(iii) Kinase assay. Kinase assays were performed by mixing soluble proteins with 50 μl of complete kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM ATP, 10 μCi of [γ-^{32}P]ATP) at 30°C for 10 min. Reaction products were resolved by SDS-PAGE on 10% gels. Proteins were visualized by staining the gels with Coomassie brilliant blue, and phosphorylated proteins were detected by autoradiography. Phosphorylated GST–E2F-1 was excised from the gel and was subjected to two-dimensional phosphopeptide mapping and phosphoamino acid analysis (see below).

Phosphorylation of E2F-1 in vivo. HeLa cells were grown to approximately 80% confluency on 10-cm^2 tissue culture dishes. Approximately 8 × 10^6 cells were washed twice with prewarmed phosphate-free DMEM. Washed cells were incubated for 3 h at 37°C in 1.5 ml of phosphate-free DMEM supplemented with 1.5% dialyzed calf serum, 2 mM glutamine, and 2 mCi of $^{32}P_i$ per ml. Labeled cells were washed with phosphate-buffered saline and lysed in 1.2 ml of NETN supplemented with 2 mM PMSF, 0.15 U of aprotinin per ml, 20 μM leupeptin, 20 μM pepstatin, 0.3 M KCl, and 5 μM microcystin. The lysates were clarified by centrifugation at 5,000 rpm for 10 min and then incubated for 1 h at 4°C with 20 μg of affinity-purified E2F-1 antibody (specific for the N-terminal 76 amino acids of E2F-1) that had been precoupled to 30 μl of a 50% slurry of Sepharose CL-4B–protein A beads. Immunoprecipitates were centrifuged, and beads were washed five times with NETN and then boiled in 60 μl of 1% SDS for 10 min. Boiled reaction mixtures were centrifuged for 10 min at 5,000 rpm. Supernatants were diluted in 1.2 ml of NETN, and E2F-1 was reimmunoprecipitated with affinity-purified E2F-1 antibody for 3 h at 4°C. Reaction mixtures were centrifuged, and beads were washed six times with NETN. Phosphoproteins were resolved by SDS-PAGE on 10% gels. Phosphorylated E2F-1 was visualized by autoradiography and was excised from the gel for mapping and for phosphoamino acid analysis.

Phosphopeptide mapping. Dried gel slices containing phosphorylated E2F-1 were soaked in 30% methanol at 37°C overnight. Gel slices were lyophilized and incubated overnight in 500 μl of a solution containing 0.01 mg of trypsin per ml in 50 mM ammonium bicarbonate at 37°C. Supernatants were lyophilized, and the tryptic digests were washed with 1 ml of distilled water and washed successively with 200 and 100 μl of 0.1 M acetic acid. Phosphopeptides were separated in the first dimension by thin-layer electrophoresis at pH 1.9. The first-dimension buffer consisted of water–acetic acid–88% formic acid in a ratio of 800/150/50. Phosphopeptides were then separated in the second dimension by ascending chromatography in a buffer consisting of *n*-butanol–pyridine–acetic acid–water in a ratio of 75/50/15/60.

Phosphoamino acid analysis. Acid hydrolysis was performed as described by Hunter and Sefton (34). The HCl was removed with three successive washes of 50, 50, and 25 μl of water. Phosphoamino acids were separated in the first dimension by thin-layer electrophoresis at pH 3.5. The first dimension buffer consisted of water-glacial acetic acid-formic acid (88% by volume) in a ratio of 897/78/25. Phosphoamino acids were then separated in the second dimension by chromatography in a buffer consisting of isopropanol-hydrochloric acid-water in a ratio of 70/15/15. The phosphoamino acid standards were visualized by staining with ninhydrin.

Electrophoretic mobility shift assays (EMSA). Sf9 cells infected with viruses encoding either GST, GST-cyclin A, GST-cyclin A/CDK2, or GST-cyclin A/CDK2$^-$(N132A) were lysed in NETN supplemented with the protease inhibitors as described above. Extracts from bacteria expressing either GST–E2F-1 or GST–DP-1 were prepared exactly as described above after lysis in NETN supplemented with protease inhibitors. The GST fusion proteins were purified on glutathione-agarose beads. Beads were washed with NETN, and the bound proteins were eluted with 20 mM glutathione in a buffer containing 200 mM Tris (pH 8.0), 0.1 M NaCl, and 1 mM EDTA. After dialysis in gel shift binding buffer (20 mM HEPES [pH 7.6], 1 mM EGTA, 10% glycerol, 0.1 M KCl, 1 mM $MgCl_2$, 0.1% [wt/vol] NP-40), protein levels were determined by the Bradford assay. The purified proteins were further analyzed by SDS-PAGE and Coomassie brilliant blue staining to ensure that equal amounts of intact proteins and/or complexes would be used in subsequent reconstitution assays.

DNA binding assays were performed exactly as described previously (63). The probe contained a single E2F site which

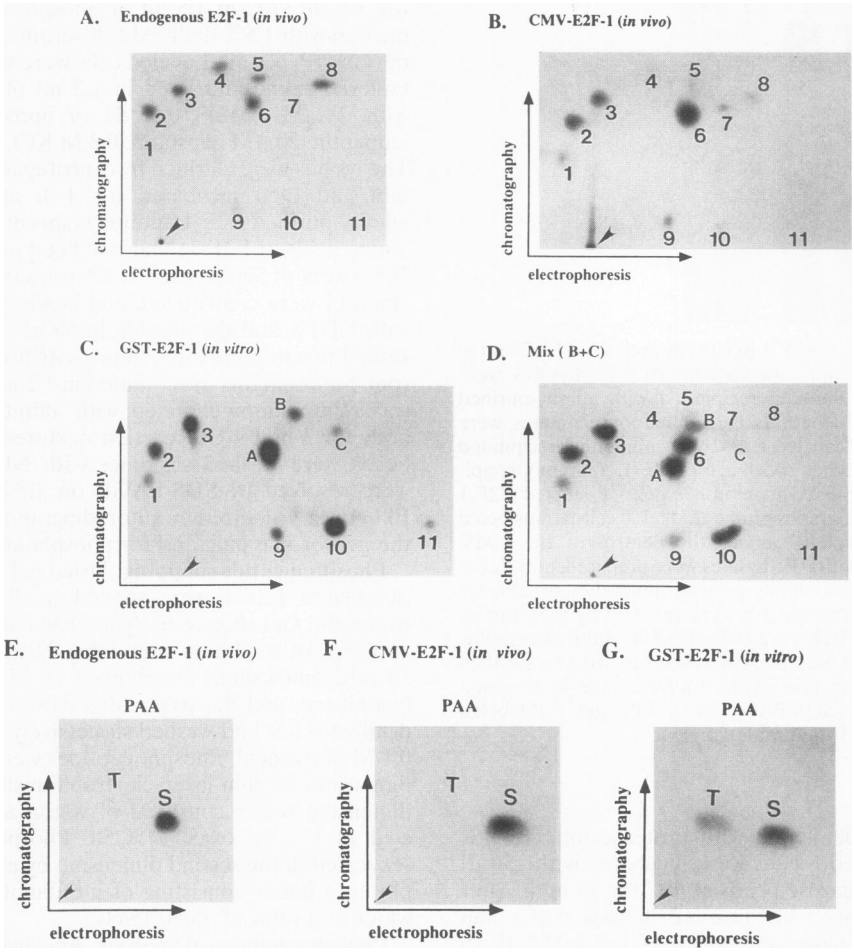

FIG. 4. Mapping of phosphorylated E2F-1 in vitro by cyclin A/CDK2 and in vivo. (A and E) HeLa cells were incubated with $^{32}P_i$, and ^{32}P-labeled E2F-1 was isolated as described for Fig. 3. ^{32}P-labeled E2F-1 was subjected both to two-dimensional phosphopeptide mapping (A) and to phosphoamino acid analysis (E). (B and F) HeLa cells, transfected with a plasmid encoding E2F-1 under the control of the CMV promoter, were incubated with $^{32}P_i$. ^{32}P-labeled E2F-1 was isolated as described for Fig. 3 and was subjected both to two-dimensional phosphopeptide mapping (B) and to phosphoamino acid analysis (F). (C and G) GST-E2F-1 was phosphorylated in vitro by GST-cyclin A/CDK2 as described for Fig. 3. ^{32}P-labeled GST–E2F-1 was subjected both to two-dimensional phosphopeptide mapping (C) and to phosphoamino acid analysis (G). (D) Mix of phosphopeptides from panels B and C. T, threonine; S, serine.

consisted of the distal site from the adenovirus E2 promoter, and 0.2 ng was used in each binding reaction. Typically, 75 ng of GST–E2F-1, GST–DP-1, or the GST–E2F-1/GST–DP-1 complex was analyzed in each assay in the presence of 50 to 400 ng of either GST, GST-cyclin A, GST-cyclin A/CDK2, or GST-cyclin A/CDK2$^-$(N132A). These quantities represent a 0.6- to 5.3-fold molar excess of the cyclin A/CDK2 complexes over E2F-1/DP-1. The complexes of E2F-1/DP-1 and cyclin A/CDK2 were assembled by incubating all components at room temperature for 30 min under DNA binding assay conditions. In some cases, ATP and/or $MgCl_2$ was added at concentrations of 500 μM and 10 mM, respectively. The protein-DNA complexes were visualized by autoradiography of a 4% nondenaturing polyacrylamide gel following electrophoresis at 400 V for approximately 1 h and 45 min.

Transient expression of E2F-1 and mutants in HeLa cells. (i) Construction of E2F-1 expression vectors. Full-length and deletion mutants of E2F-1 were expressed from the cytomegalovirus (CMV) promoter (gift of Eseng Lai). The salient features of this vector are the CMV promoter, the simian virus 40 small-T-antigen polyadenylation signal, and a pGem-1 polylinker. The E2F-1 sequences were excised from GST–E2F-1 (in pGEX-2TK; gift of Bill Kaelin) by digestion with *Bam*HI and *Eco*RI. Because the cloning site in the CMV vector is *Eco*RI, the *Bam*HI site was converted to an *Eco*RI site. The 1.4-kb E2F-1 insert was then removed by *Eco*RI digestion and then ligated into the CMV vector to generate CMV–E2F-1.

The N282 E2F-1 deletion was constructed by digestion of GST–E2F-1 (in pGEX-2TK) with *Bam*HI and *Bgl*II. Both sites were converted to *Eco*RI sites by ligation of a *Bam*HI-to-*Eco*RI adapter oligonucleotide and inserted into the *Eco*RI site of the CMV vector. A translation termination linker (5′ CTAGTCTAGACTAG 3′) was inserted into the *Xba*I site of the CMV expression vector to ensure correct expression of all truncated E2F-1 constructs. The modified N282 E2F-1 was then ligated into the CMV vector containing the translation termination signals. The N124 E2F-1 deletion was constructed by digestion of CMV–E2F-1 with *Sma*I followed by religation. The N76 E2F-1 deletion was constructed by digestion of CMV-N282 with *Dra*III and *Sma*I followed by religation.

The CMV–GST–E2F-1 and mutant constructs were derived

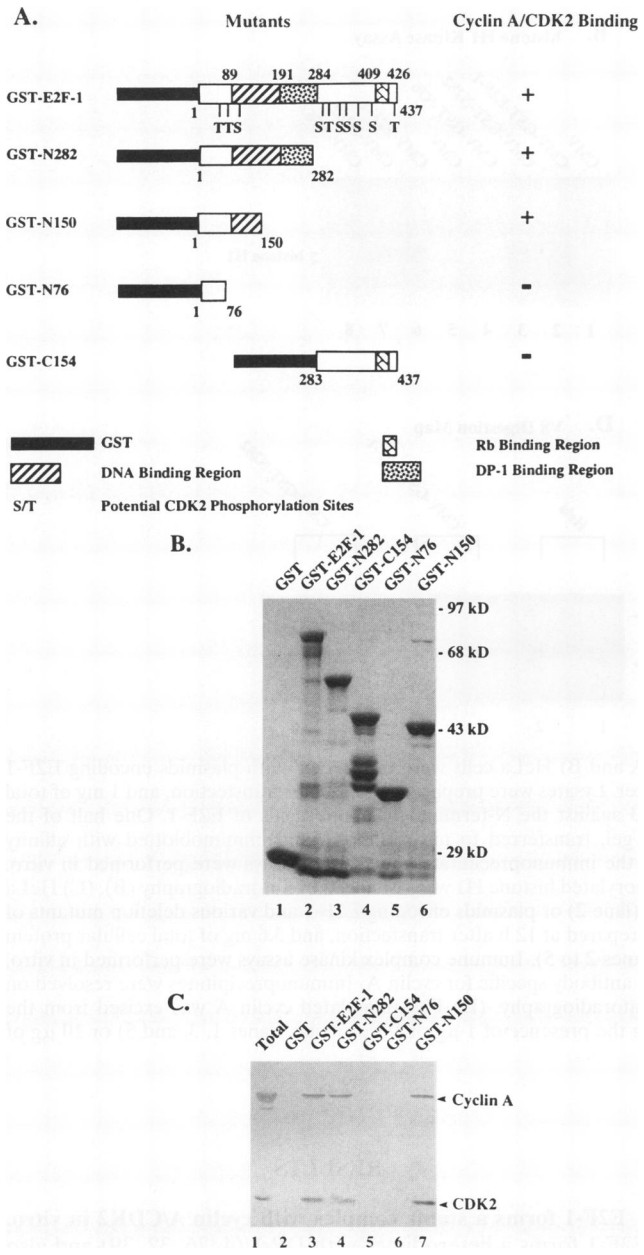

FIG. 5. The amino terminus of E2F-1 is required for stable interactions between E2F-1 and cyclin A/CDK2. (A) Schematic representation of the deletion mutants of E2F-1 generated in this study and their ability to stably associate with cyclin A/CDK2 complexes in vitro. (B) E2F-1 deletion mutants were expressed in bacteria as GST fusion proteins and were purified by using glutathione-agarose. Bound proteins were resolved on an SDS–12% polyacrylamide gel and were visualized by staining with Coomassie brilliant blue. (C) Lysates were prepared from insect cells coinfected with recombinant viruses encoding cyclin A and CDK2. Fifty micrograms of the lysate was resolved directly on an SDS–10% polyacrylamide gel (lane 1). Alternatively, 50 μg of the lysate was incubated with either GST (lane 2), GST–E2F-1 (lane 3), GST-N282 (lane 4), GST-C154 (lane 5), GST-N76 (lane 6), or GST-N150 (lane 7). Bound proteins were resolved on an SDS–10% polyacrylamide gel. Proteins were transferred to nitrocellulose and were probed with serum specific for either cyclin A (top) or CDK2 (bottom).

from E2F-1 that had been cloned into a modified pGEX-2T in which the *Eco*NI site (5′ to the start of GST) was converted to an *Xba*I site (1). After conversion of the *Xba*I site into an *Eco*RI site by linker ligation (5′ CTAGGGAATTCC 3′), GST–E2F-1 was isolated as a 2-kb *Eco*RI fragment and then ligated into the CMV vector described above. CMV-GST-N282 was constructed by digestion of CMV–GST–E2F-1 with *Bgl*II and *Xba*I and religation. CMV-GST-N76 was constructed by digestion of CMV–GST–E2F-1 with *Dra*III and *Sma*I followed by religation.

(ii) Expression of E2F-1 proteins. Plasmids encoding E2F-1 and various deletions were introduced into HeLa cells by the BES method (10, 55). Approximately 20 μg of plasmid was transfected per 10-cm^2 dish. Lysates were prepared as described below.

Interactions of cyclin A/CDK2 and E2F-1 in vivo. (i) Histone H1 kinase assays. HeLa cells were mock transfected or were transfected with 20 μg of the following plasmid: CMV, CMV–E2F-1, CMV-N282, CMV-N124, or CMV-N76. The corresponding GST–E2F-1 plasmids were also transfected. Each 10-cm^2 plate of transfected cells was lysed in 1 ml of NETN supplemented with 0.3 M KCl and protease inhibitors. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min in a microcentrifuge. In each immunoprecipitation reaction, 1 mg of lysate, 10 μg of affinity-purified E2F-1 antiserum (purified against the N-terminal 76 amino acids of E2F-1), and 15 μl of packed Sepharose CL-4B–protein A beads were used. Reaction mixtures were incubated at 4°C for 1 h. After five washes with NETN, the beads were resuspended in 1 ml of NETN and divided into two aliquots. For quantitation and normalization of E2F-1 protein levels, one aliquot was used for an immunoblot with E2F-1 antiserum. The second aliquot was washed in incomplete kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol), and an immune complex kinase assay was performed in the presence of incomplete kinase buffer supplemented with 10 μg of histone H1, 10 μM ATP, and 10 μCi of [γ-^{32}P]ATP (>4,000 Ci/mmol) for 10 min at 30°C. The reaction was terminated by the addition of SDS sample buffer. Reactions products were resolved by SDS-PAGE, and histone H1 phosphorylation was monitored by autoradiography.

(ii) Coimmunoprecipitation of E2F-1 and cyclin A. HeLa cells were mock transfected or were transfected with 20 μg of the following plasmid: CMV, CMV–GST–E2F-1, CMV-GST-N282, or CMV-GST-N76. Each 10-cm^2 plate of transfected cells was lysed in 1 ml of NETN supplemented with 0.3 M KCl and protease inhibitors. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min in a microcentrifuge. Immunoprecipitation reactions were performed by the addition of either affinity-purified E2F-1 antiserum (purified against the N-terminal 76 amino acids of E2F-1) or 4.5 μg of cyclin A antiserum (C160, precoupled to 3.6 μg of donkey anti-mouse immunoglobulin G [heavy plus light chain; Jackson Laboratories]) and 45 μl of packed Sepharose CL-4B–protein A beads. Immunoprecipitates were washed five times in NETN and then twice in incomplete kinase buffer. For quantitation and normalization of E2F-1 protein levels, one aliquot was used for an immunoblot with E2F-1 antiserum. An immune complex kinase assay was performed with the second aliquot in the presence of incomplete kinase buffer supplemented with 10 μM ATP and 10 μCi of [γ-^{32}P]ATP (>4,000 Ci/mmol) for 10 min at 30°C. After three washes with NETN, the immunoprecipitates were boiled in 60 μl of 1% SDS for 5 to 10 min. Reactions were clarified, and the supernatant was transferred to a fresh tube and diluted into 1.2 ml of NETN. Reimmunoprecipitation reactions were performed in the presence of 4.5 μg of cyclin A antiserum (C160, precoupled to 3.6 μg of

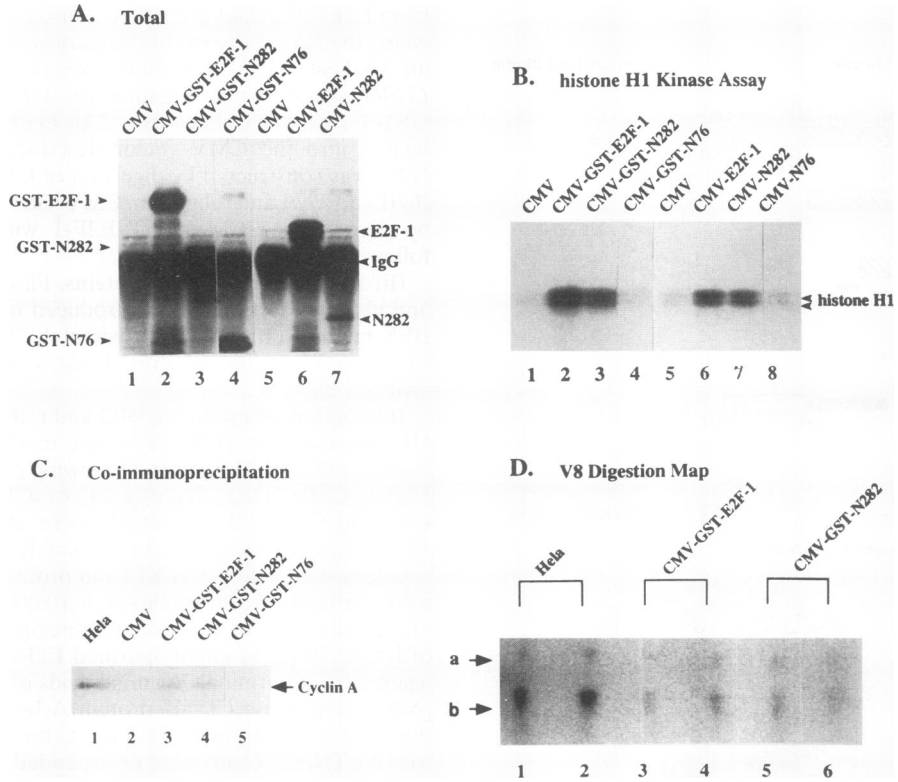

FIG. 6. Interaction between E2F-1 and cyclin A/CDK2 in HeLa cells. (A and B) HeLa cells were transfected with plasmids encoding E2F-1 and various deletion mutants of E2F-1 under the control of the CMV promoter. Lysates were prepared at 12 h after transfection, and 1 mg of total cellular protein was incubated with antibody that had been affinity purified against the N-terminal 76 amino acids of E2F-1. One half of the immunoprecipitate was resolved directly on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with affinity purified E2F-1 antibody (A). Histone H1 was added to the second half of the immunoprecipitate, and kinase assays were performed in vitro. Reaction products were resolved on an SDS–10% polyacrylamide gel. Phosphorylated histone H1 was visualized by autoradiography (B). (C) HeLa cells were mock transfected (lane 1) or were transfected with plasmid alone (lane 2) or plasmids encoding E2F-1 and various deletion mutants of E2F-1 under the control of the CMV promoter (lanes 3 to 5). Lysates were prepared at 12 h after transfection, and 3.6 mg of total cellular protein was incubated with antibody specific for either cyclin A (lane 1) or E2F-1 (lanes 2 to 5). Immune complex kinase assays were performed in vitro. Reaction mixtures were washed, boiled in 1% SDS, and then incubated with antibody specific for cyclin A. Immunoprecipitates were resolved on an SDS–10% polyacrylamide gel. ^{32}P-labeled cyclin A was visualized by autoradiography. (D) Phosphorylated cyclin A was excised from the SDS-gel shown in panel C (lanes 1, 3, and 4). V8 mapping was performed in the presence of 1 μg of V8 protease (lanes 1, 3, and 5) or 10 μg of V8 protease (lanes 2, 4, and 6).

donkey anti-mouse immunoglobulin G (heavy plus light chain) and 45 μl of packed Sepharose CL-4B–protein A beads. After incubation at 4°C for 1 h, the beads were collected and washed with NETN. Immunoprecipitates were resolved by SDS-PAGE, and ^{32}P-labeled cyclin A was visualized by autoradiography.

(iii) V8 mapping. Phosphorylated cyclin A was excised from SDS-gels and was incubated in mapping buffer (50 mM Tris [pH 6.8], 0.1% SDS, 1 mM EDTA, 10% glycerol) for 30 min at room temperature. The gel slices were then inserted into the wells of an SDS–20% polyacrylamide gel and were overlaid with 10 μl of mapping buffer supplemented with 1 mg of bovine serum albumin per ml, an additional 10% glycerol, and bromphenol blue. Ten microliters of V8 protease (suspended in mapping buffer at a concentration of either 0.1 or 1 μg/μl) was added to the top of each well. The gel was run at 25 mA until the bromphenol blue dye reached the center of the stack. The power was then turned off for 30 min and then restarted at 40 mA until the bromphenol blue dye reached the bottom of the gel. The gel was dried immediately, and the phosphopeptides were visualized with the use of a PhosphorImager.

RESULTS

E2F-1 forms a stable complex with cyclin A/CDK2 in vitro. E2F-1 forms a heterodimer with DP-1 (4, 26, 32, 39) and also exists in a complex with cyclin A and CDK2 during S phase (5, 18, 47, 50, 59). To understand the functional consequences of these interactions, we have developed a system in which the interactions can be studied in vitro with purified components. E2F-1 and DP-1 were overproduced in bacteria as a GST fusion proteins, and cyclin A and CDK2 were overproduced in insect cells by using a baculovirus expression system. E2F-1 was examined for its ability to interact with cyclin A alone, with CDK2 alone, or with cyclin A/CDK2 complexes. As shown in Fig. 1 and 2, significant complex formation was not detected between E2F-1 and cyclin A alone (Fig. 1, lane 4; Fig. 2A, lanes 7 to 12) or with CDK2 alone (Fig. 1, lane 5; Fig. 2B, lanes 7 to 12). However, interactions with E2F-1 were greatly facilitated when complexes between cyclin A and CDK2 were assayed (Fig. 1, lane 6; Fig. 2C, lanes 7 to 12). Therefore, the affinity of E2F-1 for the cyclin A/CDK2 complex was significantly higher than for either cyclin A or CDK2 alone. Unlike E2F-1, DP-1

was unable to bind to cyclin A, CDK2, or cyclin A/CDK2 complexes in vitro (Fig. 1, lanes 7 to 9).

E2F-1 is a phosphoprotein in HeLa cells and is a substrate for cyclin A/CDK2 in vitro. To examine the phosphorylation state of E2F-1 in vivo, labeling experiments were performed in HeLa cells both in the absence (Fig. 3, lane 1) and in the presence (Fig. 3, lane 2) of E2F-1 overexpression. An affinity-purified antibody purified against the N-terminal 76 amino acids of E2F-1 was used. Sequences within the N terminus of various members of the E2F family of transcription factors are highly divergent; thus, this antibody is predicted to be specific for E2F-1. As seen in Fig. 3, E2F-1 was phosphorylated in HeLa cells (lanes 1 and 2). Immunoprecipitations with preimmune sera were negative (data not shown). To test whether E2F-1 was a substrate for cyclin A/CDK2, kinase assays were performed in vitro. As seen in Fig. 3 (lane 4), E2F-1 was readily phosphorylated by cyclin A/CDK2 in vitro. ^{32}P-labeled E2F-1 was digested with trypsin, and the phosphopeptides were resolved in two dimensions (Fig. 4). Several phosphopeptides were detected in each case. Interestingly, several of the phosphopeptides derived from E2F-1 labeled in vivo comigrated with the phosphopeptides derived from E2F-1 labeled in vitro (Fig. 4D). Phosphoamino acid analysis revealed primarily phosphoserine subsequent to phosphorylation in vivo (Fig. 4E and F), whereas both phosphoserine and phosphothreonine were detected subsequent to phosphorylation in vitro (Fig. 4G).

Stable ternary complexes between E2F-1, cyclin A, and CDK2 do not require kinase-active cyclin A/CDK2. To determine whether the kinase activity of the CDK2/cyclin A complex was important for the stable association between CDK2/cyclin A and E2F-1, a kinase-deficient mutant of CDK2 was assayed. This mutant, CDK2$^-$(N132A), contains an alanine substitution for asparagine at position 132. Asn-132 is invariant in protein kinases, and the crystal structure of CDK2 demonstrated that it participates in the coordination of Mg^{2+}. CDK2$^-$(N132A) assembles with cyclin A but is inactive as a protein kinase (14). As seen in Fig. 2C and D, there were no detectable differences in the ability of E2F-1 to associate with complexes of either cyclin A/CDK2$^-$(N132A) or cyclin A/wild-type CDK2. Identical results were obtained when a kinase-deficient mutant of CDK2 in which lysines 33 and 34 were replaced with serine and threonine, respectively, was used (data not shown). These results indicate that the kinase activity of the complex does not contribute to the stability of the E2F-1/cyclin A/CDK2 complex.

The N terminus of E2F-1 is required for cyclin A/CDK2 binding. To localize regions required for cyclin A/CDK2 binding, a series of E2F-1 deletion mutants were generated (Fig. 5A). The mutant proteins were expressed in bacteria as GST fusion proteins and were purified on glutathione-agarose beads (Fig. 5B). Recombinant proteins were tested for the ability to bind to cyclin A/CDK2 (Fig. 5C). As seen in Fig. 5C, the C-terminal region of E2F-1, which contains the binding site for RB family members, failed to interact with cyclin A/CDK2 (lane 5), but the N-terminal region (N282) interacted efficiently (lane 4). To further delineate the boundaries within the N terminus of E2F-1 required for cyclin A/CDK2 binding, fusion proteins containing the first 150 amino acids (N150) and the first 76 amino acids (N76) were also generated and tested in the binding assay described above. As shown in Fig. 5C, N150 interacted with cyclin A/CDK2 (lane 7), but N76 failed to bind to cyclin A/CDK2 (lane 6). Taken together, these experiments localize the binding region to sequences within the first 150 amino acids of E2F-1.

The N terminus of E2F-1 is sufficient for cyclin A/CDK2 binding in vivo. The results described above indicated that E2F-1 could form a complex with cyclin A/CDK2 in vitro through a direct interaction within its N terminus. To determine whether stable complex formation between E2F-1 and cyclin A/CDK2 could also occur in vivo in the absence of RB or RB-like partners, transient transfection assays were performed. In this case, C-terminal truncations of E2F-1 that lacked the binding site for RB and RB-like partners were used. The association of cyclin A/CDK2 with E2F-1 was then assayed by scoring for the coprecipitation of either CDK2 activity (by histone H1 kinase assays; Fig. 6A and B) or of cyclin A protein (Fig. 6C and D) with the E2F-1 deletion mutants.

E2F-1 and two deletion mutants were expressed in HeLa cells under the control of the CMV promoter. The two deletion mutants were N282 (which contains binding sites for the cyclin A/CDK2 complex, for DNA, and for DP-1) and N76 (which contains none of these regions). E2F-1 and the deletion mutants were also expressed as GST fusion proteins. From results obtained in vitro, cyclin A/CDK2 was predicted to associate with full-length E2F-1 and N282 but not with N76.

To compare the abilities of E2F-1 and the deletion mutants to interact with cyclin A/CDK2 in vivo, it was critical that the immune complexes contain comparable levels of E2F-1 protein. Thus, a portion of each immunoprecipitate was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with an antibody specific for E2F-1. As seen in Fig. 6A, the overall levels of E2F-1 and the deletion mutants were comparable. Because of the small size of N76, the non-GST-fused form could not be detected in this manner. The remaining portion of each immunoprecipitate was tested for its ability to phosphorylate histone H1. As seen in Fig. 6B, histone H1 kinase activity specifically coprecipitated with full-length E2F-1 (lanes 2 and 6) and N282 (lanes 3 and 7), but failed to coprecipitate with N76 (lanes 4 and 8) and the vector control (lanes 1 and 5). Furthermore, the background of histone H1 kinase activity due to endogenous E2F-1 was low (lanes 1 and 5), indicating that the enhanced H1 kinase activity associated with full-length E2F-1 and N282 was not due to endogenous E2F-1.

To look specifically for an association between E2F-1 and cyclin A, E2F-1 and associated mutants were expressed in HeLa cells. The E2F-1 immunoprecipitates were then examined for the coprecipitation of cyclin A with E2F-1. Specifically, recombinant E2F-1 proteins were immunoprecipitated with affinity-purified E2F-1 antibody, and kinase assays were performed in vitro. ^{32}P-labeled proteins were then dissociated by boiling in SDS and incubated with a monoclonal antibody specific for cyclin A. As seen in Fig. 6C, a phosphoprotein of 48 kDa coprecipitated with full-length GST-E2F-1 (lane 3) and N282 (lane 4) but failed to co-precipitate with N76 (lane 5) or with the CMV control (lane 2). This 48-kDa protein specifically comigrated with phosphorylated cyclin A (lane 1), and V8 mapping studies demonstrated that it was cyclin A (Fig. 6D).

Interactions between E2F-1 and cyclin A/Cdk2 in vivo are independent of DP-1. The results described above indicated that E2F-1 could form a complex with cyclin A/CDK2 in vitro in the absence of RB family members. To determine whether stable complex formation between E2F-1 and cyclin A/CDK2 could also occur in vivo in the absence of DP-1, transient transfection assays were performed (Fig. 7). In addition to the mutants described above, an additional mutant of E2F-1 containing the N-terminal 124 amino acids (N124) that lacked binding sites for both DP-1 and RB family members was used. E2F-1, N282 (which contains the DP-1-binding region), and N124 (which lacks DP-1-binding region) were expressed in HeLa cells under the control of the CMV promoter. Cell lysates were prepared and immunoprecipitated with affinity-

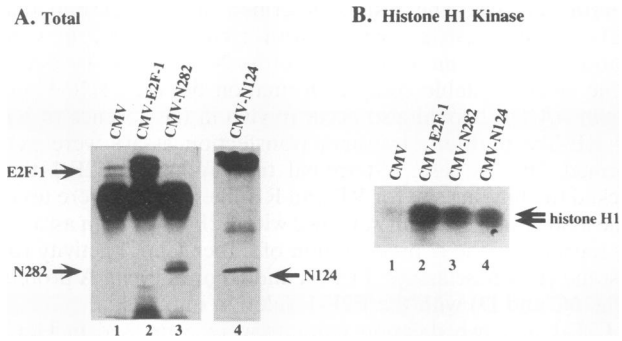

FIG. 7. Interaction between E2F-1 and cyclin A/CDK2 is independent of DP-1 in vivo. (A and B) HeLa cells were transfected with plasmids encoding vector alone, E2F-1, and the indicated deletion mutants of E2F-1 under the control of the CMV promoter. Lysates were prepared at 12 h after transfection, and 1 mg of total cellular protein was incubated with antibody that had been affinity purified against the N-terminal 76 amino acids of E2F-1. One half of the immunoprecipitate was resolved directly on either an SDS–10% (lanes 1 to 3) or SDS–20% (lane 4) polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with affinity-purified E2F-1 antibody. The SDS–20% gel was necessary for visualization of the smaller N124 protein (A). Histone H1 was added to the second half of the immunoprecipitate, and kinase assays were performed in vitro. Reaction products were resolved on an SDS–10% polyacrylamide gel. Phosphorylated histone H1 was visualized by autoradiography (B).

purified E2F-1 antibody. One half of each immunoprecipitate was analyzed for the expression of E2F-1 proteins by immunoblotting (Fig. 7A); the other half was assayed for histone H1 kinase activity (Fig. 7B). From results obtained in vitro, and assuming that interactions were independent of DP-1, stable associations of cyclin A/CDK2 were predicted to occur with

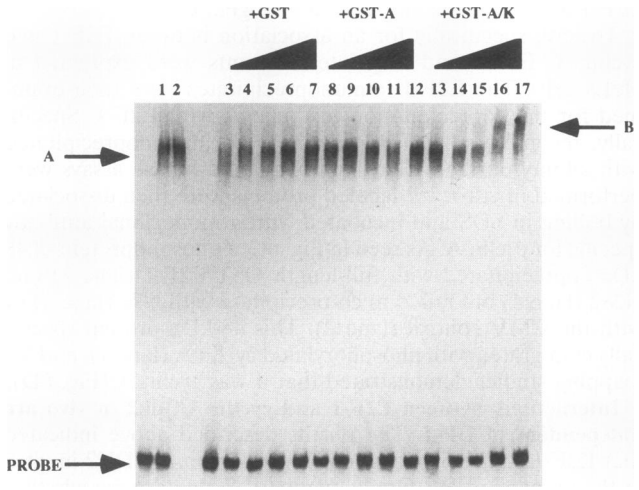

FIG. 8. EMSA for interaction of purified E2F-1/DP-1 and cyclin A/CDK2 complexes. The EMSA for E2F was performed exactly as described in Materials and Methods. The probe consisted of the distal E2F site from the adenovirus E2 promoter. The E2F-1/DP-1 complex was reconstituted under standard EMSA conditions, using 75 ng each of purified GST–E2F-1 and GST–DP-1. Duplicate lanes are shown, and the complex is indicated (complex A, lanes 1 and 2). Either purified GST (lanes 3 to 7), GST-cyclin A (lanes 8 to 12), or GST-cyclin A/CDK2 (lanes 13 to 17) was tested. In each set, either 50, 100, 200, 300, or 400 ng of added protein was used. The complex of E2F-1, DP-1, cyclin A, and CDK2 is indicated (complex B).

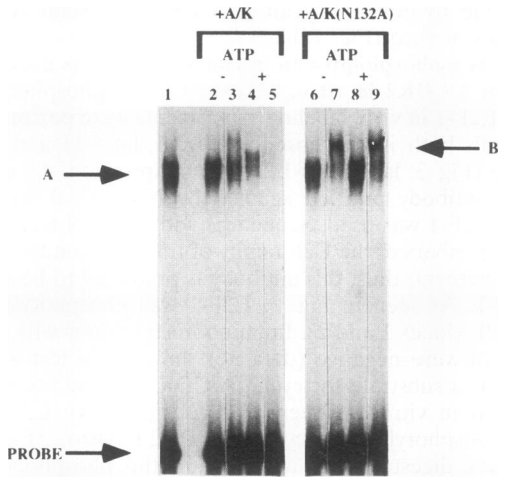

FIG. 9. Kinase-active cyclin A/CDK2 inhibits the DNA-binding ability of the E2F-1/DP-1 complex. The E2F-1/DP-1 complex was reconstituted under standard EMSA conditions, using 75 ng each of purified GST–E2F-1 and GST–DP-1 (lane 1). Either purified GST cyclin A/CDK2 (lanes 2 to 5) or GST-cyclin A/CDK2$^-$(N132A) (lanes 6 to 9) was also included. In each set, 50 ng (lanes 2, 4, 6, and 8) or 300 ng (lanes 3, 5, 7, and 9) of complexes was used. To examine the effects under kinase conditions (lanes 4, 5, 8, and 9), 10 mM $MgCl_2$ and 500 μM ATP were included in the standard EMSA reaction. To examine the effects under control conditions (lanes 2, 3, 6, and 7), 10 mM $MgCl_2$ was included in the standard EMSA reaction. Analysis of DNA binding proceeded as described in Materials and Methods.

E2F-1, N282, and N124. In Fig. 6B, N76 (which lacks the DP-1-, DNA-, and RB-binding regions) was defective in association of histone H1 kinase activity (lanes 4 and 8). As predicted, histone H1 kinase activity specifically coprecipitated with full-length E2F-1 (Fig. 7B, lane 2), N282 (Fig. 7B, lane 3), and N124 (Fig. 7B, lane 4) but not with the vector control (Fig. 7B, lane 1).

The purified complex of E2F-1, DP-1, cyclin A, and CDK2 can bind DNA. The effects of cyclin A/CDK2 interactions on the DNA-binding ability of the E2F-1/DP-1 complex were tested by using purified components in a standard EMSA. The probe contained a single E2F site composed of the distal E2F site of the adenovirus E2 promoter (63, 64). GST-cyclin A and GST-cyclin A/CDK2 were purified from overproducing insect cells, and GST–E2F-1 and GST–DP-1 were purified from bacteria. Reconstitution proceeded under standard E2F EMSA conditions.

The ability of E2F-1 to bind to DNA is enhanced upon heterodimerization with DP-1. As shown in Fig. 8 (lanes 1 and 2, complex A), the complex of GST–E2F-1 and GST–DP-1 could be efficiently reconstituted and exhibited good binding to the E2F DNA probe, in good agreement with published results (4, 26, 32, 39). However, the addition of neither GST nor GST-cyclin A had any significant effect on the E2F-1/DP-1 complex, ruling out any artifactual interactions through the GST moiety of the fusion proteins (Fig. 8, lanes 3 to 12). However, when purified GST-cyclin A/CDK2 (~0.7- to 5.3-fold molar excess over GST–E2F-1/DP-1) was reconstituted with purified GST–E2F-1/DP-1, a new complex (B) of reduced mobility was observed (Fig. 8, lanes 16 and 17). In addition, purified kinase-deficient GST-cyclin A/CDK2$^-$(N132A) could also be reconstituted efficiently with the GST–E2F-1/DP-1 complex (Fig. 9, lanes 7 and 9). In this assay, the binding affinity of E2F-1/DP-1 for the kinase-deficient complex [GST-

cyclin A/CDK2$^-$(N132A)] was approximately fourfold higher than for the kinase-active complex (GST-cyclin A/CDK2) (data not shown). The molecular basis for this difference in affinity is not yet clear. Nonetheless, it is apparent that kinase activity was not required for stable and direct association of cyclin A/CDK2 with E2F-1/DP-1 in a DNA-binding complex

Kinase-active cyclin A/CDK2 inhibits the DNA-binding ability of the E2F-1/DP-1 complex. We next tested the effects of phosphorylation by cyclin A/CDK2 on the DNA-binding ability of the E2F-1/DP-1 factor (Fig. 9). In each reaction, 75 ng each of purified GST–E2F-1 and of GST–DP-1 were used (Fig. 9, lane 1, complex A). Reactions were performed both under kinase conditions (10 mM $MgCl_2$ and 500 μM ATP) and under control conditions (10 mM $MgCl_2$). Either 50 or 300 ng of cyclin A/CDK2 was used. The kinase-deficient mutant of cyclin A/CDK2$^-$(N132A) was used as a control. As seen in Fig. 9, kinase-active cyclin A/CDK2 reduced the ability of E2F-1/DP-1 to bind to DNA under kinase conditions in the presence of ATP (Fig. 9; compare lanes 4 and 5 with lanes 2 and 3). Under kinase conditions, approximately fourfold or complete reduction in DNA binding was observed in the presence of 50 or 300 ng of cyclin A/CDK2, respectively. The kinase-deficient mutant of cyclin A/CDK2$^-$(N132A) had no effect on the DNA-binding ability of E2F-1/DP-1 under either condition (Fig. 9, lanes 6 to 9). In contrast to stable association (Fig. 8), kinase activity of cyclin A/CDK2 was required for the inhibition of the DNA-binding ability of E2F-1/DP-1 upon phosphorylation.

DISCUSSION

In this report, we have examined the interactions between E2F-1, DP-1, cyclin A, and CDK2. To do so, we have developed a system for examining protein-protein interactions that uses recombinant proteins. We have determined that E2F-1 binds to the cyclin A/CDK2 complex through a domain within its N terminus. E2F-1 contains a transcriptional activation domain within its C terminus, and a binding site for RB and RB-like partners is nestled within this domain (at amino acids 409 to 426). A DNA-binding domain is located within its N-terminal region (residues 89 to 191) (15, 25, 37). Following the DNA-binding domain is a hydrophobic heptad repeat (residues 199 to 234) that is important for heterodimerization with DP-1 (4, 26, 39). The cyclin A/CDK2-binding site is distinct from each of these sites. A deletion mutant of E2F-1 containing only the N-terminal 150 amino acids of E2F-1 (which lacks DP-1- and RB-binding domains) bound stably to cyclin A/CDK2 in vitro. A deletion mutant of E2F-1 containing only the N-terminal 124 amino acids of E2F-1 (which lacks DP-1- and RB-binding domains) bound stably to cyclin A/CDK2 in vivo. A larger deletion containing only the N-terminal 76 amino acids of E2F-1 did not bind to cyclin A/CDK2 when tested in vitro or in vivo. We conclude from these studies that amino acids bordered by residues 76 and 124 are critical for cyclin A/CDK2 binding, but we have not precisely defined the borders of the binding domain. Given that the E2F-1/cyclin A/CDK2 complex still bound to DNA, cyclin A/CDK2 binding probably does not overlap the DNA-binding domain. Furthermore, because the complex could be reconstituted from purified components, the interactions of cyclin A/CDK2 with E2F-1/DP-1 are direct. Thus, this region represents a new functional region of E2F-1 which allows direct coupling between E2F-1 and cell cycle regulatory kinases.

Interestingly, neither the cyclin A subunit nor the CDK2 subunit alone formed a stable complex with E2F-1. These results are similar to those of our previous studies which demonstrated that complex formation between cyclin A and CDK2 is a prerequisite for stable interactions with p107 (51). Cyclins regulate their cognate CDKs in several ways. Cyclin binding induces conformational changes that allow phosphorylation of the CDK by regulatory kinases (both stimulatory and inhibitory), and cyclins target their respective CDKs to appropriate intracellular substrates. We have determined that both E2F-1 (this study) and p107 (51) are substrates for cyclin A/CDK2 in vitro. Given the high degree of overlap in the phosphopeptides detected subsequent to phosphorylation of E2F-1 and p107 in vitro by cyclin A/CDK2 with those detected in vivo, it is likely that both E2F-1 and p107 are substrates for cyclin A/CDK2 in vivo as well. A formal possibility remains that other CDK family members may also phosphorylate E2F-1 in vivo, although only CDK2 has been demonstrated to stably associate with E2F-1 in vivo. Thus, stable substrate-kinase interactions prevail in the case of both E2F-1 and p107. In the case of p107, stable quaternary complexes composed of an E2F family member (distinct from E2F-1) and kinase-active cyclin/CDK complexes (composed of either cyclin E/CDK2 or cyclin A/CDK2) are assembled in a cell cycle-specific manner. p107 contains distinct sites for cyclin A/CDK2 and E2F-X binding. In this case, p107 may facilitate the interactions between cyclin A/CDK2 and its respective E2F species. Because E2F-1 directly interacts with cyclin A/CDK2, the binding of p107 may be dispensable for targeting of the kinase to E2F-1.

Phosphorylation has been shown to regulate transcription factor function at several levels, including intracellular compartmentalization, DNA-binding activity, and/or transcriptional activation (for a review, see reference 33). The association of an active CDK2 kinase with E2F-1 provides the possibility for regulatory interactions at several levels. At the promoter level, it is plausible that phosphorylation could modulate the assembly of initiation complexes by affecting the activities of general transcription factors (for a review, see reference 66). At the level of E2F regulation, phosphorylation may modulate the protein-protein interactions between E2F-1 and RB, p130, and/or DP-1. For example, the binding of RB to E2F-1 is inhibitory to E2F-1 function (3, 9, 11, 21, 23, 24, 27, 28, 31, 49, 53, 54, 62). Phosphorylation of RB leads to dissociation of the E2F-1/RB complex, and RB is a substrate for the CDK kinase family (30). Direct phosphorylation of E2F-1 may also regulate the E2F-1–RB interactions. If phosphorylation of E2F-1 leads to RB dissociation, then phosphorylation would effectively stimulate E2F function.

We have demonstrated that the DNA-binding capacity of E2F-1/DP-1 is inhibited following phosphorylation by cyclin A/CDK2 kinase. Heterodimerization of E2F-1 and DP-1 stimulates E2F-1-dependent transcription (4, 26, 39). Intriguingly, DP-1 did not bind cyclin A/CDK2, so E2F-1 must bring the kinase to the complex. The inhibitory effect of phosphorylation on the DNA-binding ability of E2F-1/DP-1 was apparent with kinase-active but not kinase-deficient CDK2. However, both cyclin A/CDK2 and cyclin A/CDK2$^-$(N132A) could bind efficiently to E2F-1/DP-1. Thus, intact kinase activity of cyclin A/CDK2 was required for inhibition of DNA binding but was not required for the stable and direct interaction with the E2F-1/DP-1 complex.

In this report, we have demonstrated that E2F-1 is a substrate for CDK2 both in vitro and in vivo. In addition, we have found that DP-1 is a substrate for cyclin A/CDK2 in vitro (unpublished data). Thus, this inhibition of DNA binding may be mediated through the phosphorylation of E2F-1 and/or DP-1. An attractive model is that phosphorylation of either

E2F-1 or DP-1 within the complex leads to loss of DNA binding by dissociation of the heterodimer. In this case, phosphorylation would play a negative role in the regulation of promoters that utilize E2F-1/DP-1. On the surface, this result appears paradoxical given that cyclin A/CDK2 binding to E2F-1 accompanies the entry of cells into S phase, a time in the cell cycle when E2F-1-regulated promoters are being turned on. However, this mechanism may explain how the transcription of E2F-1-dependent genes is turned off as cells exit from S phase. One prediction of this model is that the cyclin A/CDK2-mediated phosphorylation of E2F-1/DP-1 (at those sites that promote complex dissociation) would occur in late S phase. Studies are currently under way to specifically test this model and to further delineate the mechanisms whereby cyclin A/CDK2 regulates E2F-1 function.

The association of a kinase with a transcription factor may be a recurring theme for transcriptional regulation. Recently, the transcription factor JUN has been shown to associate stably with the JNK-1 kinase, which phosphorylates JUN in potential regulatory sites (17). Furthermore, JNK-1 is a member of the mitogen-activated protein kinase family and may allow coupling of transcriptional events with regulation of cell proliferation. The association of pivotal transcription factors such as E2F-1 and JUN with kinases contributes to the growing complexity of cell cycle and transcriptional regulatory events.

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ADDENDUM IN PROOF

While the manuscript was under review, a similar study was published by Krek et al. (W. Krek, M. Ewen, S. Shirodkar, Z. Arany, W. Kaelin, and D. Livingston, Cell **78**:161–172, 1994). A related study was also published by Dynlacht et al. (B. Dynlacht, O. Flores, J. A. Lees, and E. Harlow, Genes Dev. **8**:1772–1786, 1994).

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