Identification of AP-2 as an interactive target of Rb and a regulator of the G1/S control element of the hamster histone H3.2 promoter

Frank Wu and Amy S. Lee*

Department of Biochemistry and Molecular Biology and the USC/Norris Comprehensive Cancer Center, University of Southern California School of Medicine, 1441 Eastlake Avenue, Los Angeles, CA 90033, USA

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ABSTRACT

Previous studies have established that a 32 bp cis-regulatory region, referred to as the H3core spanning –241 to –210 of the hamster histone H3.2 promoter, is critical for its G1/S-phase induction of transcription. Here we report that the transcription factor AP-2 is a major component of the protein complex which interacts with the H3core from hamster nuclear extracts. In search of the control mechanism(s) whereby AP-2 can mediate cell cycle regulation of the histone H3.2 promoter, we found that AP-2 can physically interact with the retinoblastoma (Rb) tumor suppressor protein in vitro, and when over-expressed, can also associate with Rb in vivo. Importantly, in contrast to the majority of Rb binding proteins, the C-terminal domain of Rb alone is sufficient for its interaction with AP-2. Using a reporter gene system linking tandem copies of the H3core to a heterologous minimal promoter, we demonstrated that over-expression of AP-2 proteins results in transactivation of the reporter gene through the H3core in a sequence-specific but orientation-independent manner. Additionally, this stimulative effect was suppressed by co-expression of Rb. Thus, AP-2, through its physical and functional interaction with Rb, may contribute to the cell cycle regulation of its target genes.

INTRODUCTION

The histone gene family encodes a group of small basic proteins (the core histones H2A, H2B, H3, H4 and the linker histone H1) that are essential for the packaging of newly synthesized DNA into nucleosomes. Expression of replication-dependent histone genes is regulated in part by transcriptional activation at the G1/S-phase boundary, coupled with the S-phase specific stabilization of the newly synthesized histone transcripts (1–3). To understand the molecular mechanisms which confer G1/S-phase induction of the mammalian histone genes, key cis-regulatory elements of a variety of histone genes have been identified in both the promoters and coding sequences (1–6).

Within a given histone gene subtype, each gene has conserved control elements. Correspondingly, different trans-acting factors for subtype specific histone gene transcription have been reported. For example, H1TF-1 interacts with a highly conserved AC-box and H1TF-2 with a CCAAT-box in the H1 promoters (7–9). In the case of H2A, it has been reported that E2F binds to a conserved E2F recognition motif in the human H2A.1 promoter and activates its promoter activity (10). For H2B, Oct-1 is a major promoter binding factor (11). For H4, Sp1 is needed for its high level transcription (12) while IRF-2 regulates its induction in S-phase (13). It has also been reported that a transcription complex termed HiNF-D containing CDC2, cyclin A, and a retinoblastoma (Rb)-related protein interacts with multiple recognition motifs in human H1, H3 and H4 promoters (14).

The H3 gene promoters ranging from wheat to human contain a highly conserved motif which resembles but differs from the AP-1 and CRE consensus binding sites (15). This motif is contained within a 32 bp region spanning –241 to –210, which by criterion of 5′ deletion, is critical for stringent G1/S-phase regulation of the hamster H3.2 promoter (4). This conserved sequence motif is implicated in the transcriptional control of both plant and vertebrate H3 genes and has been shown to be a nuclear factor binding site (15–18). Using site-directed mutagenesis, we have demonstrated a 10 bp region spanning –240 and –231 is required for the S-phase dependent increase of the hamster H3.2 transcription in cells synchronized by serum starvation or aphidicolin block (15,19). Further in vitro analysis has shown that the 3′ flanking sequence of this 10 bp region is also required for the formation of a protein complex which binds to a 40 bp H3.2 promoter subfragment spanning –250 to –211, referred to below as the H3core (15,19). Using nuclear extracts prepared from serum synchronized cells at various stages of the cell cycle in gel mobility shift assays, we have observed cell cycle fluctuation of the H3core binding activities (19). The H3core complex contains a minor component of AP-1, and a major protein species distinct from AP-1 as a high affinity H3core binding factor which is previously unidentified (19).

AP-2, a 52 kDa nuclear protein, is a retinoic acid-inducible and developmentally regulated transcription factor (20–25). AP-2 has a unique dimerization motif and binds to DNA as a dimer (26,27). Both transcriptional activation and repression effects have been

*To whom correspondence should be addressed. Tel: +1 323 865 0507; Fax: +1 323 865 0094; Email: amylee@zygote.hsc.usc.edu
postulated to act through AP-2 (28–35). AP-2 exerts its critical function in regulating gene expression in response to a number of different signal transduction pathways (22,23,32,36). Since the discovery of AP-2, three new isoforms (AP-2B, AP-2β and AP-2β′AP-2.2) have been identified and shown to form dimers (20,37,38). One of them, AP-2B, is a naturally occurring transdominant-negative mutant which lacks the C-terminal DNA-binding domain but retains its dimerization ability with AP-2 (20). For clarity, the originally discovered AP-2 is referred to below as AP-2A. Interestingly, AP-2A activity can be modified with the nuclease treated rabbit reticulocyte lysate system in vivo from 1 μg of linearized DNA templates by using the T3 or T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX). Radiolabeled proteins were translated in vitro with the nuclease treated rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of [35S]methionine (20 μCi per 25 μl of reaction volume). AP-2 and cyclin D2 proteins were stored in the presence of 1 mM Na3VO4, 10 μg/ml leupeptin and 1 μg/ml aprotinin at −70°C.

**Glutathione S-transferase (GST) pull-down assays**

The GST-proteins used were as follows: GST, GST-AP-2A (gifts of Dr P. Kannan, University of Texas at Houston), GST-AP-2β (BDβ) (gift of Dr R. Buettner, University of Regensburg, Germany) containing only the C-terminal DNA binding domain (BDβ) of the murine AP-2β, GST-TBP and GST-Rb(379–928) (gifts of Dr D. Dean, Washington University), GST-Rb-A/B(379–792) and GST-Rb-C(768–928) (gifts of Dr J. Y. Wang, University of California, San Diego), GST-Rb-B/C(646–928) (gift of Dr Y. K. Fung, University of Southern California), GST-c-Myc(259–439) (gift of Dr K. Calame, Columbia University), and GST-H-Ras (gift of Dr D. Broek, University of Southern California). Following expression of GST-proteins in Escherichia coli and IPTG induction, GST-proteins, prepared by sonication in PBS plus 1% Triton X-100, were purified by affinity chromatography with glutathione-linked agarose beads (Sigma, St Louis, MO), and the protein yields were verified by Coomassie Blue staining. Equal amounts of in vitro translated and 35S-labeled AP-2A, AP-2β or cyclin D2 proteins (4 μl of each) were mixed with bacterially expressed GST-proteins bound onto beads (∼25 pmol of each). Then the reaction mixtures were incubated in 50 μl of the binding buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM Na3VO4 and 0.5% NP-40) in the presence of 1 mg/ml bovine serum albumin (BSA), at 4°C with gentle rocking for 2–4 h. The beads were then washed five times each with 800 μl of NETN buffer (20 mM Tris–HCl, pH 8, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40). Proteins bound on beads were boiled in sodium dodecyl sulfate (SDS) sample buffer for 5 min, subjected to 10% SDS–PAGE, and detected by autoradiography.

**Transfection assays**

Conditions for transfection into the hamster fibroblast K12 cells were described (43). The AP-2 expression vectors pSV/hAP-2A and pSV/hAP-2B were gifts of Drs M. Tainsky and P. Kannan (University of Texas at Houston); pCMV/hAP-2A and pCMV/hAP-2β were gifts of Dr R. Buettner (University of Regensburg, Germany); the full-length Rb (pHβ-Apr-I-Rb) and its vector (pHβ-Apr-I-neo) were gifts of Dr Y. K. Fung (University of Southern California, Los Angeles, CA). All transfection reaction mixtures contained either 3 μg of pCMV-lacZ, pRSV-lacZ, or 5 μg of grp78-lacZ (containing 2 kb of the rat grp78 promoter fused to the lacZ gene) which served as an internal control of transfection efficiency.
Immunoprecipitation and western analysis

K12 cells were transiently co-transfected with pCMV/mAP-2β and pHβ-Apr-1-Rb using SuperFect reagent (Qiagen, Hilden, Germany). After 48 h, the cells were lysed in ELB buffer (50 mM HEPES, pH 7.0, 250 mM NaCl and 0.1% NP-40) in situ at 4°C for 10 min. The cell lysate was diluted in ELB buffer containing a final concentration of 125 mM NaCl, and precleared by incubating with 50 µl mixture of Protein-A Sepharose CL-4B (Sigma, St Louis, MO) and Protein-G Sepharose-4FL beads (Pharmacia-LKB, Piscataway, NJ) at 4°C for 1 h. Precleared lysates were immunoprecipitated with either normal rabbit or mouse serum (Sigma, St Louis, MO), anti-AP-2 or anti-Rb (IF8) (Santa Cruz, CA), or anti-v-Src (Oncogene, Cambridge, MA) antibody at 4°C for 4–6 h with gentle rocking. Protein complexes were collected by incubation with either Protein-A Sepharose CL-4B or Protein-G Sepharose-4FL beads at 4°C for 2 h followed by a quick spin. Protein complexes bound onto beads were washed in 1 ml of ice-cold buffer containing 50 mM Tris–HCl, pH 7.4, 125 mM NaCl and 0.1% NP-40 five times and resolved by a 10% SDS–PAGE. The western analysis was performed by using an anti-Rb antibody [1:500 dilution in T-TBS (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20) containing 2% BSA] and the antigenic protein was detected by an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL).

RESULTS

The H3core contains an AP-2-like binding site directly 3’ to an AP-1-like site

The sequence of the H3core spanning –250 to –211 is shown in Figure 1A. Previously, it has been shown that the 10 bp region spanning –240 to –231 is required for S-phase induction of the H3.2 promoter and that mutation of this sequence, corem (Table 1) resulted in the loss of binding affinity for the H3core complex (15,19). Within this region, there is an H3 AP-1-like element (CGAGTCA) which differs from the consensus AP-1 binding site as shown in Figure 1B. The sequence spanning –240 to –231 is mutated such that both the AP-1-like site and one base of its adjacent AP-2-like site are affected; 5’AP-1 and 3’AP-2 sites are marked by the thin and thick boxes, respectively. Arrows indicate the guanine residues protected by DNA methylation interference assays (19). The sequence of the H3core spanning –250 to –211 is identical by a black square.

Localization of the AP-2 binding site on the H3core

To map the AP-2 binding site within the H3core, mutated forms of the H3core oligonucleotide were synthesized (Table 1). The oligomer corem is identical to the wild-type H3core except that the sequence spanning –240 to –231 is mutated such that both the AP-1-like site and one base of its adjacent AP-2-like site are affected; 5’mcore is mutated from –250 to –241, and AP-2mcore from –230 to –215 to destroy all AP-2-like GC-rich sequence motifs. As controls, the consensus site for AP-1 or AP-2, and a mutated form of the AP-2 consensus site (AP-2m) were also used as competitors (Table 1). The EMSAs were performed using CHO nuclear extracts and radiolabeled H3core as probe. For comparison, radiolabeled consensus AP-2 site was also used in a similar competition assay.

First, our results indicated that the AP-2 binding site resides within the 3’ half of the H3core since the 5’m core competed for the H3core complex as efficient as the wild-type H3core (Fig. 3A). Second, within the 3’ half of the H3core, the AP-2 binding site is mapped within –240 to –215 since this is the sequence mutated in both corem and AP-2’mcore, which resulted in the loss of ability to compete for the H3core complex (Fig. 3A). Subsequent mutation of the AP-1 site within this sequence further narrowed the binding region of the H3core to further 3’ (Fig. 3A).
The H3 AP-1 and H3 AP-2 binding sites are indicated by the thin and thick boxes, respectively. The mutated sequences are highlighted and are in italics. The consensus sequences for the other known binding sites are indicated by the dashed boxes. The linker sequences at the end of the oligomers are indicated by lower case letters.

Table 1. DNA sequences of the synthetic oligomers used in EMSAs

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence</th>
<th>Ref.</th>
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<tr>
<td>H3core</td>
<td>ctagACGGAACCTTCGGACTCCCGCCGCGGCTGGACAA tCATCCTGGAGAACCCTTCGGCTGGACAA</td>
<td>(15)</td>
</tr>
<tr>
<td>core*</td>
<td>ctagACGGAACCTTTGaaggccGGCTGGACAA</td>
<td>(15)</td>
</tr>
<tr>
<td>5′core</td>
<td>ctagATATCGTcggccCGCTGGACAA</td>
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<tr>
<td>AP-1′core</td>
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<td>This study</td>
</tr>
<tr>
<td>AP-2′core</td>
<td>ctagACGGAACCTTCGGACTCCCGCCGCGGCTGGACAA tCATCCTGGAGAACCCTTCGGCTGGACAA</td>
<td>This study</td>
</tr>
<tr>
<td>AP-2</td>
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<td>(22,25)</td>
</tr>
<tr>
<td>AP-1</td>
<td>agctAAAGCGAACTTCGGACTCCCGCCGCGGCTGGACAA</td>
<td>(61,62)</td>
</tr>
<tr>
<td>Sp1</td>
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<tr>
<td>Ets-1</td>
<td>ctagACGGAACCTTCGGACTCCCGCCGCGGCTGGACAA</td>
<td>(65)</td>
</tr>
<tr>
<td>E2F</td>
<td>ctagACGGAACCTTCGGACTCCCGCCGCGGCTGGACAA</td>
<td>(66)</td>
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The H3 AP-1 and H3 AP-2 binding sites are indicated by the thin and thick boxes, respectively. The mutated sequences are highlighted and are in italics. The consensus sequences for the other known binding sites are indicated by the dashed boxes. The linker sequences at the end of the oligomers are indicated by lower case letters.

AP-2 binding from −234 to −215 (Fig. 4). Using the consensus AP-2 binding site as probe, the formation of the AP-2 complex was efficiently inhibited by the homologous AP-2 site as well as by the H3core and the 5′core (Fig. 3B). This is in contrast with the H3core probe where competition by the consensus AP-2 site, even at high molar ratios, was not as efficient as the H3core or the 5′core. Under these conditions, a residual complex with an electrophoretic mobility similar to that of the AP-1 complex was detected (Figs 2 A and 3 A). Neither core, AP-2core, AP-2m, nor AP-1 binding sites were effective as competitors for the AP-2 complex.

To dissociate AP-1 and AP-2 protein binding on the H3core, two approaches were used. First, AP-1 protein was removed by binding site competitions and second, the AP-1-like binding site was eliminated by mutation. In the first approach, with H3core as probe in EMSAs, AP-2 protein binding was abolished by an anti-AP-2 antibody (Fig. 3C, lanes 2 and 3), or by molar excess of the consensus AP-2 site (Fig. 3C, lanes 10 and 11); while the major AP-2 complex was eliminated, a residual complex remained under these conditions. Thus, further removal of the factor binding to the AP-1-like sequence by site competition prior to the addition of anti-AP-2 antibody should further reduce the H3core complex. Indeed, preincubation of the CHO nuclear extracts with a 25-fold molar excess of the consensus AP-1 site, followed by addition of anti-AP-2 antibody, resulted in much reduction of the H3core complex (Fig. 3C, lanes 4 and 5). In contrast, the addition of control rabbit serum or anti-GRP78 antibody had no effect (Fig. 3C, lanes 6–9).

In the second approach, AP-1 binding activity was eliminated completely by mutation of the AP-1-like site in the H3core to generate an AP-1mcore used in EMSA (Table 1). In this case, the major complex formed with the AP-1mcore probe is AP-2 since competition by molar excess of the consensus AP-2 site alone completely eliminated the complex, whereas the AP-2m site had no effect (Fig. 4A). The absence of AP-1 binding activity was confirmed since no competition was observed when molar excess of the AP-1 site was used as competitor. Further, addition of anti-AP-2 antibody, but not the normal rabbit serum nor anti-GRP78 antisera, in EMSAs performed with the AP-1mcore as probe resulted in near complete elimination of the AP-2 complex (Fig. 4B). The efficiency and specificity of the anti-AP-2 antibody was confirmed by the specific blockage of the formation of the AP-2 complex using the consensus AP-2 site as probe (Fig. 4C). Collectively, these data indicate that the binding of AP-2 to the H3core is independent of the AP-1 binding activity. Further, using recombinant AP-1 and AP-2 protein in EMSAs, we observed that both factors could co-occupy the H3core (data not shown).
Figure 2. In vitro binding of AP-2 to the H3core. (A) EMSAs were performed with 2 µg of CHO nuclear extracts (CHO NE), 1 ng of H3core as probe (asterisk) and 200 ng of poly dI·dC as non-specific competitor. A 25- or 50-fold molar excess of the various binding site competitors as indicated on top was used. The sequence of the competitor oligomers are shown in Table 1. The positions of the minor AP-1 complex (open arrowhead) and the major AP-2 complex (closed arrowhead) are indicated. (B) EMSAs were performed using 1 ng of bacterially expressed recombinant human AP-2A (rAP-2) (Promega, Madison, WI), 1 ng of the H3core as probe and 200 ng of poly dI·dC. The AP-2 complexes were competed against a 25- or 50-fold molar excess of the consensus wild-type or mutated AP-2 (AP-2<sup>m</sup>) binding site.

Interaction of AP-2 with Rb in vitro and in vivo

To elucidate the mechanism whereby AP-2 can affect the transcription of the replication-dependent H3.2 gene, we examined whether AP-2 associates with the cell cycle regulatory molecules. Previously, it has been reported that Rb can repress the transactivation activity of a truncated AP-2A in the form of a fusion protein (39). To test whether there is direct physical interaction between AP-2 and Rb, [35S]methionine-labeled AP-2A and AP-2β were prepared by in vitro transcription and translation. The radiolabeled proteins were then mixed with bacterially expressed GST-AP-2A, GST-Rb(379–928) or GST protein alone. The GST-Rb(379–928) fusion protein contains a truncated N-terminal end but retains the functionally important pocket and C-terminal domains. The GST-bound proteins were resolved on SDS–PAGE and detected by autoradiography. While confirming the previous observations that AP-2A and AP-2β can form homo- or hetero-dimeric complexes, this in vitro assay demonstrated that both AP-2 proteins can interact with Rb (Fig. 5).

To map the interaction domains between Rb and AP-2, different GST-proteins were expressed in E.coli, affinity-purified, and their concentration verified by SDS–PAGE and Coomassie Blue staining (data not shown). In vitro GST pull-down assays were performed with 35S-labeled and in vitro transcribed/translated full-length (Fig. 6) or N-terminal AP-2 fragments (Fig. 7). The GST-proteins tested included the GST alone, GST-Rb(379–928), GST-Rb-A/B containing the small pocket domain, GST-Rb-B/C containing both the B and C-terminal domains, GST-Rb-C containing only the C-terminal domain and the unrelated H-Ras protein. Equal amounts of the GST proteins were mixed with the radiolabeled AP-2 proteins or murine cyclin D2 as a positive control. Strikingly, unlike the majority of Rb binding proteins, the C-terminal domain of Rb alone was sufficient to bind either

Figure 3. Mapping of the AP-2 binding site within the H3core. EMSAs were performed using CHO nuclear extracts (CHO NE), using either the H3core (A and C) or the consensus AP-2 binding site (B) as probe (asterisk). The complexes were competed with a 25- or 50-fold molar excess of binding site competitors as indicated on top. In (A and C), the positions of the minor AP-1 (open arrowhead) and the major AP-2 (closed arrowhead) complexes are indicated. In (B), the position of the AP-2 complex is indicated. In (C), the effects of various antisera (1.5 or 3 µg) and/or binding site competitors (25- or 50-fold molar excess) on the formation of the AP-1 and AP-2 complexes are shown. The α-AP-2 (C18) is an affinity-purified, rabbit polyclonal antibody against a carboxyl peptide of human AP-2A and is crossreactive with AP-2β (Santa Cruz Biotechnology, Santa Cruz, CA). NRS is normal rabbit serum, and α-78 is a rabbit polyclonal anti-hamster GRP78 peptide antibody. The antibodies were pre-incubated with the nuclear extracts on ice for 15 min prior to the addition of probes. In lanes 4–9, a 25-fold molar excess of the consensus AP-1 binding site was added to the reaction prior to the addition of the antisera as indicated on top.

Figure 4. Effect of AP-1 site mutation on the formation of the H3core complex. EMSAs were performed using CHO NE and the AP-1<sup>m</sup>core or consensus AP-2 site as probe (asterisk). (A) The complexes were competed with a 25– or 50-fold molar excess of binding site competitors as indicated on top. (B) The formation of the complex was competed with increasing amounts (1.5 and 3 µg) of the antisera as indicated. The position of the AP-2 complex is indicated. (C) The EMSA was performed same as (B) except the consensus AP-2 site was used as probe.
AP-2A (Fig. 6A) or AP-2β (Fig. 6B). Further, both AP-2A and AP-2β also interacted with GST-Rb-A/B containing the small pocket domain although the interaction was less strong than that observed with the GST-Rb-C or GST Rb-B/C. In case of cyclin D2 which has been reported to bind to the Rb-C-terminal domain (44), it bound to Rb(379–928), Rb-B/C and Rb-C but not to Rb-A/B as expected (Fig. 6C).

To map the region of AP-2 necessary for Rb interaction, AP-2A and AP-2β protein fragments, containing the N-terminal 262 and 252 amino acids respectively, were used in GST pull-down assays (Fig. 7). Radiolabeled AP-2 fragments or cyclin D2 was mixed with equal amounts of GST alone, GST-AP-2A, AP-2β(DBD), c-Myc(259–439), Rb(379–928), TBP or H-Ras. In contrast to the full-length protein, AP-2A(N262) devoid of the C-terminal dimerization domain was unable to dimerize with itself or AP-2β, and unable to bind Rb (Fig. 7A). The same results were observed with AP-2β(N252) (Fig. 7B). The lack of binding to Rb could not be due to non-functional Rb used in this experiment since cyclin D2 could bind as expected (Fig. 7C). Therefore, the N-terminal portion of AP-2 alone was insufficient for the interaction with Rb.

To determine whether AP-2 and Rb can associate in vivo, co-immunoprecipitation experiments were performed. For this purpose, K12 fibroblasts were co-transfected with expression vectors for AP-2β and Rb. The cell lysates were first immunoprecipitated with either normal rabbit or mouse serum, anti-AP-2, anti-Rb or anti-Src antibody. The immunoprecipitates were then resolved on SDS–PAGE, and western blotted against the anti-Rb antibody. A protein band with molecular weight of ~105–110 kDa was detected by the anti-Rb antibody in immunoprecipitates of anti-AP-2 and anti-Rb but not by the normal control serum or anti-Src antibody (Fig. 8). The intense band detected in lanes 1–4 represented a mixture of immunoglobulins (Ig) from the rabbit polyclonal antibodies or control sera and in lanes 5–8, specific Ig from mouse monoclonal antibodies. The extra smaller bands detected in lanes 5–6 were probably degradation products of Rb. Therefore, AP-2 and Rb, when over-expressed, can associate with each other in vivo.

Figure 5. Interaction of AP-2 proteins with Rb in vitro. GST pull-down assays were performed with in vitro translated and 35S-labeled (A) AP-2A or (B) AP-2β. Lane 1, 10% of the input radiolabeled protein used for the assay; lane 2, the 35S-labeled AP-2 protein was mixed with GST; lane 3, with GST-AP-2A; and lane 4, with GST-Rb(379–928). The proteins bound onto the GST-beads were eluted, applied to a 10% SDS–PAGE, and detected by autoradiography. The positions of the protein size markers (in kDa) run in parallel are indicated.

Figure 6. Mapping the interaction domains of Rb with AP-2. GST pull-down assays were performed with GST-proteins and 35S-labeled full-length (A) AP-2A, (B) AP-2β or (C) cyclin D2 proteins. Lanes 1 and 8, 10% of the input protein; lane 2, GST alone; lane 3, GST-Rb(379–938); lane 4, GST-Rb-A/B(379–792); lane 5, GST-Rb-B/C(646–928); lane 6, GST-Rb-C(768–928); and lane 7, GST-H-Ras. Arrows indicate the positions of input or interacting proteins.

Figure 7. AP-2A and AP-2β protein fragments, containing the N-terminal 262 and 252 amino acids respectively, were used in GST pull-down assays. Lanes 1 and 8, 10% of the input radiolabeled protein used for the assay; lane 2, GST alone; lane 3, GST-Rb(379–928); lane 4, GST-Rb-A/B(379–792); lane 5, GST-Rb-B/C(646–928); lane 6, GST-Rb-C(768–928); and lane 7, GST-H-Ras. Arrows indicate the positions of input or interacting proteins.
cancer cell growth through the activation of the p21WAFl/CIP1. AP-2 acts as a negative regulator of c-Myc (30), and inhibits growth factors on fibroblast growth (51). Other reports imply that factor binding protein-5, which can potentiate the effect of insulin on fibroblasts, AP-2-mediated transactivation contributes to the hormone gene and activates its transcription (50). In human example, AP-2 can bind the promoter of the human growth factor and cellular cell cycle regulators such as the SV40 large T antigen (34), E1A (35) and the transcription factor c-Myc (30). In addition, both AP-2 and AP-2β have been shown to be over-expressed in cancer cell lines (48), and are able to transform cells when over-expressed (20,48,49). AP-2 binding sites have been identified in the promoters of a wide variety of cellular genes associated with cell growth and apoptosis. In various gene and cell systems, AP-2 is able to exert a wide range of effects, through both activation and repression of specific gene activity. For example, AP-2 can bind the promoter of the human growth hormone gene and activate its transcription (50). In human fibroblasts, AP-2-mediated transactivation contributes to the constitutively high expression of the human insulin-like growth factor binding protein-5, which can potentiate the effect of insulin growth factors on fibroblast growth (51). Other reports imply that AP-2 acts as a negative regulator of c-Myc (30), and inhibits cancer cell growth through the activation of the p21WAFl/CIP1 expression (52). The N-ras oncogene causes AP-2 transcriptional self-interference which leads to transformation (49). In mammary carcinoma cells, when c-erbB-2 is over-expressed, the AP-2 protein is expressed at elevated levels (48). Further, E1A-mediated repression of a matrix metalloproteinase gene implicated in tumor metastasis has been correlated with its ability to bind and inactivate AP-2 (35). Recently, it was reported that loss of AP-2 results in up-regulation of a cell surface glycoprotein MCAM and an increase in tumor growth and metastasis of human melanoma cells (53). Nonetheless, as many promoters which contain an AP-2 site are not regulated in a growth-dependent manner, it is evident that the mechanism for the AP-2-mediated regulation of cellular promoters is complex and likely to involve other co-factors and regulatory signals unique to each gene system (30,32,33).

DISCUSSION

Histone and other replication-dependent genes have provided important model systems for studying the control of gene expression during the cell cycle (45,46). Our investigation into the factors interacting with the H3core, the G1/S control element of the hamster H3.2 promoter, led to the discovery that AP-2 is a major DNA binding component. Further, we showed here that AP-2 is a novel target for Rb, and its regulation of the H3core transcriptional activity can be modulated by Rb.

While AP-2 is clearly important in mediating gene expression during embryonic morphogenesis and adult cell differentiation (40–42,47), increasingly, AP-2 has been linked to the cell cycle control and tumor progression. AP-2 interacts with both viral and cellular cell cycle regulators such as the SV40 large T antigen (34), E1A (35) and the transcription factor c-Myc (30). In addition, both AP-2A and AP-2β have been shown to be over-expressed in cancer cell lines (48), and are able to transform cells when over-expressed (20,48,49). AP-2 binding sites have been identified in the promoters of a wide variety of cellular genes associated with cell growth and apoptosis. In various gene and cell systems, AP-2 is able to exert a wide range of effects, through both activation and repression of specific gene activity. For example, AP-2 can bind the promoter of the human growth hormone gene and activate its transcription (50). In human fibroblasts, AP-2-mediated transactivation contributes to the constitutively high expression of the human insulin-like growth factor binding protein-5, which can potentiate the effect of insulin growth factors on fibroblast growth (51). Other reports imply that AP-2 acts as a negative regulator of c-Myc (30), and inhibits cancer cell growth through the activation of the p21WAFl/CIP1 expression (52). The N-ras oncogene causes AP-2 transcriptional self-interference which leads to transformation (49). In mammary carcinoma cells, when c-erbB-2 is over-expressed, the AP-2 protein is expressed at elevated levels (48). Further, E1A-mediated repression of a matrix metalloproteinase gene implicated in tumor metastasis has been correlated with its ability to bind and inactivate AP-2 (35). Recently, it was reported that loss of AP-2 results in up-regulation of a cell surface glycoprotein MCAM and an increase in tumor growth and metastasis of human melanoma cells (53). Nonetheless, as many promoters which contain an AP-2 site are not regulated in a growth-dependent manner, it is evident that the mechanism for the AP-2-mediated regulation of cellular promoters is complex and likely to involve other co-factors and regulatory signals unique to each gene system (30,32,33).

In search of possible mechanism(s) whereby AP-2 can contribute to regulation of cell cycle gene expression, we found that AP-2 is a target of Rb. Through mapping of the interaction domains of these two proteins, we further determined that in contrast to the majority of Rb binding proteins, the C-terminal domain of Rb alone is sufficient for AP-2 binding. This novel finding is potentially significant since it implies that the binding of AP-2 to Rb may resemble that of E2F, a key regulator of cell cycle (54,55). While our manuscript was in preparation, it was reported that Rb and c-Myc activation of E-cadherin gene expression in epithelial cells acted through interaction with AP-2 (47). Studies of Batsche et al. (47) showed that the N-terminal domain of AP-2 and the oncoprotein binding domain and the C-terminal domain of Rb are required for the interaction. Our analysis demonstrated that the N-terminal portion of AP-2 may be required but is not sufficient for its binding to Rb (Fig. 7). Importantly, under our experimental conditions, we were able to demonstrate that the C-terminal domain of Rb, spanning 768–928 amino acids, was sufficient to bind AP-2. In agreement with their results, we also observed that the Rb-A/B domain is able to bind AP-2 in vitro and that Rb and AP-2 can be co-immunoprecipitated in vivo. Thus, these results indicate that AP-2 is a new interactive target of Rb.

In the histone H3.2 promoter, the H3core sequence is required for its G1/S regulation. Here we provide evidence that AP-2 is a major binding component of the H3core complex. Through
protein–protein interaction. Rb could be recruited onto the H3core and cooperate with AP-2 to regulate H3.2 expression during the cell cycle. Depending on the promoter, Rb can both activate or suppress gene expression through interaction with other regulatory proteins. Suppression of transcription by Rb can be achieved through sequestration of activating transcription factors (46,56,57), or by binding and inactivating transcription factors at the promoter (39). Furthermore, for some specific genes, Rb can also repress transcription through its interaction with histone deacetylase 1 (58). Additionally, Rb-mediated suppression of AP-2 activity through the H3core may involve changes in post-translational modifications of these proteins. Future investigations into Rb regulation of AP-2 activity will address these important issues.

The diverse and pleiotrophic effect of AP-2 on transcription strongly argues it is likely to be cell-type and promoter specific. It has been shown that in complex cell cycle regulated promoters, the function of a particular control element is highly dependent on its interaction with other control elements as well as the basal transcriptional machinery (59). The H3.2 promoter is also highly complex and contains multiple positive and negative regulatory elements with opposing activities adjacent to the H3core (60). The mechanisms whereby AP-2 and Rb act in concert with the other regulatory factors to regulate H3.2 expression remain to be determined. Nonetheless, our findings that AP-2 binds to the G1/S regulatory domain of a replication-dependent H3.2 promoter and interacts with Rb provide a novel mechanistic explanation for a plausible role of AP-2 in cell cycle control.

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