Interaction between HMGA1 and Retinoblastoma Protein Is Required for Adipocyte Differentiation*

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It is generally accepted that the regulation of adipogenesis prevents obesity. However, the mechanisms controlling adipogenesis have not been completely defined. We have previously demonstrated that HMGA1 proteins play a critical role in adipogenesis. In fact, suppression of HMGA1 protein synthesis by antisense technology dramatically increased growth rate and impaired adipocyte differentiation in 3T3-L1 cells. Furthermore, we showed that HMGA1 strongly potentiated the capacity of the CCAAT/enhancer-binding protein β (C/EBPβ) transcriptional factor to transactivate the leptin promoter, an adipocyte-specific promoter. In this study we demonstrate that HMGA1 physically interacts with retinoblastoma protein (RB), which is also required in adipocyte differentiation. Moreover, we show that RB, C/EBPβ, and HMGA1 proteins are down- and up-regulated, respectively. We also show that RB regulates CDC25A and CDC6, two cell cycle proteins induced by E2F-1 protein, by displacing HDAC1 from their promoters, or by using Hmga1−/− embryonic stem (ES) cells. Further, all HMGA1 proteins in adipocytes display different DNA binding activities during adipocyte differentiation and that HMGA1 strongly potentiates C/EBPβ transcriptional activity during adipocyte differentiation (10). Together these data demonstrate a regulatory role of HMGA1 on adipocyte differentiation and a role in the adipogenic process.

The HMGA proteins, including HMGA1a, HMGA1b, and HMGA2, are chromatinic proteins that do not have transcriptional activity per se but are able to regulate the transcription of several genes by protein/DNA and protein/protein interactions (1–7). They have an important role in the process of adipogenesis. In fact, the targeting of the Hmga2 gene causes a drastic reduction in fat tissue, whereas its activation by the deprivation of its carboxyl-terminal box results in large accumulation of fat tissue in ectopic areas (8, 9). Moreover, we have shown that suppression of the HMGA1 protein in 3T3-L1 cells stimulates the C/EBPβ DNA binding activity during adipocyte differentiation and that HMGA1 strongly potentiates C/EBPβ to transactivate the leptin promoter, an adipocyte-specific promoter (10).

It is known that transcriptional regulation of adipocyte differentiation requires the concerted activity of several transcription factors, which control growth arrest as well as the coordinated expression of adipocyte-specific genes. The retinoblastoma proteins are thought to be critical in controlling cell cycle and terminal differentiation. It has been described that RB is able to induce cell cycle arrest by negatively regulating the E2F family of transcriptional factors (15–16). RB also controls terminal differentiation by binding to and regulating the activity of several tissue-specific trans-activators (17). It has also been demonstrated that RB and C/EBPβ physically interact and functionally cooperate to activate several promoters during adipogenesis (18). Moreover, it has been shown that HMGA2

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The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; HA, hemagglutinin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ES, embryonic stem; RA, all-trans-retinoic acid; EMSA, electrophoretic mobility shift assay; EB, embryoid body; HDAC1, histone deacetylase 1; RB, retinoblastoma protein.
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interacts with RB and induces E2F1 activity in mouse pituitary adenomas by displacing HDAC1 from the RB-E2F1 complex (19). These data prompted us to investigate the hypothesis that HMGA1 proteins might also bind the RB proteins giving rise to a multiprotein complex able to regulate adipocyte differentiation.

Here we report that HMGA1 protein physically interacts with RB during adipocyte differentiation. This interaction is direct because in vitro translated RB binds GST-HMGA1.

Moreover, we show that RB, C/EBPβ, and HMGA1 proteins cooperate in controlling the Id1 and the obese promoter activity, which are down-regulated and up-regulated, respectively, during adipocyte differentiation. Additionally, we demonstrate that the interaction between HMGA1 and RB is important in the regulation of CDC25A and CDC6 promoters, which are also controlled by the E2F-1 protein, by displacing HDAC1 from the RB-E2F1 complex.

EXPERIMENTAL PROCEDURES

Cell Cultures—The mouse NIH 3T3-L1 cells used in this study were generously donated by Dr. E. Santos (NCI, National Institutes of Health). Cell cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (Invitrogen). Induction of adipocyte differentiation in 3T3-L1 cells was essentially performed as described elsewhere (20). Briefly, confluent 3T3-L1 cells were cultured in DMEM supplemented with 10% calf serum up to the late log phase. Two days later, they were grown in DMEM containing 10% fetal calf serum and 0.5 mM 1-methyl-3-isobutylxanthine, 10−6 m dexamethasone, and 10−6 m insulin for 48 h. Cells were further cultured in the same culture medium devoid of dexamethasone and insulin. The adipocytes were maintained in DMEM containing 10% fetal calf serum (Invitrogen).

Plasmids—To construct the expression plasmid (pCMV/Hmga1b), the full-length Hmga1b cDNA was cloned into the HindIII site of pCMV-Tag 1b (Invitrogen). To construct the expression plasmids containing the entire and various portions of the Hmga1 coding sequence, PCR with pairs of primers linked to restriction sites (EcoRI and BamHI) and the size and purity of the bound proteins were evaluated by SDS-polyacrylamide gel. The recombinant proteins were eluted with a buffer containing PBS, 10 mM imidazole, and 10% (v/v) glycerol. For the His-tagged RB protein, the supernatant was purified by using nickel-agarose beads supplied with the His-Trap purification kit (Amersham Biosciences) following the manufacturer’s instructions, eluted with 500 mM imidazole, and dialyzed in PBS. Equal amounts of GST and GST-Hmga1b proteins (5 μg) were used for binding assays. For the RB recombinant protein, transcription and translation reactions were performed with the T7-rabbit reticulocyte lysate kit (Promega, Madison, WI), as suggested by the manufacturer. The in vitro translated RB was allowed to associate with glutathione-agarose-bound GST or GST-Hmga1b for 2 h in lysis buffer at 4 °C (18). The pellets were washed four times in lysis buffer; the proteins were dissociated by boiling in loading buffer and electrophoresed on SDS-7.5% polyacrylamide gel. The gel was dried and autoradiographed. The HDAC1 full-length recombinant protein was from Abnova (P01).

Immunoblotting and Immunoprecipitation—Nuclear extracts were prepared as follows. Cells were washed twice in PBS and resuspended in 10 volumes of a solution containing 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 0.5 mM DTT (homogenization solution). The cells were disrupted.
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by passage through a 26-gauge needle. Nuclei were collected by centrifugation at 1500 rpm and resuspended in 1.2 ml of extraction solution containing 10 mM Hepes, pH 7.9, 0.4 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol to allow elution of nuclear proteins by gentle shaking at 4 °C. Nuclei were pelleted again by centrifugation at 12,000 rpm, and the supernatant was stored at −70 °C until use. The protease inhibitors leupeptin (5 mM), aprotinin (1.5 mM), phenylmethylsulfonyl fluoride (2 mM), pepstatin A (3 mM), benzamidine (1 mM) were added to both homogenization and extraction solutions.

Total protein extracts from human 293 cells were prepared with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with protease inhibitors on ice for 15 min. Total extracts from terminally differentiated or undifferentiated NIH 3T3-L1 fibroblasts were prepared as described previously (18). Protein concentration was determined by the Bradford protein assay (Bio-Rad). The antibodies used for immunoblotting, Western blotting, and ChIP were as follows: anti-C/EBP β (C-19) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HA 12CA5 mouse monoclonal antibodies (Roche Applied Science); anti-RB (C-15) rabbit polyclonal antibodies (Santa Cruz Biotechnology); anti-E2F1 (C-20) rabbit polyclonal antibodies (Santa Cruz Biotechnology); anti-acetylated E2F1 polyclonal antibody was raised against a synthetic E2F1 peptide acetylated at position 117, 120, and 125 after conjugation to keyhole limpet hemocyanin (24); anti-HDAC1 (06-720) rabbit polyclonal antibodies (Upstate Biotechnology Inc., Lake Placid, NY); anti-HMGA1 polyclonal antibody was raised against a synthetic peptide located in the NH2-terminal region (25, 26). To confirm equal loading, the same Western blots were incubated with antibodies against 

**RT-PCR Analysis**—Total RNA was extracted by RNAzol (Tel-Test, Inc., Friendswood, TX). 1 μg of total RNA, digested with free-RNase DNase, was reverse-transcribed using random hexanucleotides as primers (100 mM) and 12 units of avian myeloblastosis virus reverse transcriptase (Invitrogen). Subsequent PCR amplification was as follows: 200 ng of cDNA were amplified in a 25-μl reaction mixture containing TaqDNA polymerase buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.4 mM of each primer, 1 unit of TaqDNA polymerase (PerkinElmer Life Sciences). The PCR amplification was performed for 25 cycles (94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min). The primers used for aP2 gene expression were 5′-GATGC-CCTTTGTTGGAACCTGG-3′ and 3′-TTTATCGAATTCCAGGCCCA-5′ (30). In addition, a set of primers specific for the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was added into the reaction after 20 cycles of PCR to serve as an internal control for the amount of cDNA tested. The GAPDH-specific primers were 5′-ACAAGTTCCATATGATGACCC-3′ and 3′-GACTCACGACGTACTCA-5′ (30). All reactions were analyzed on a 2% agarose gel and visualized using ethidium bromide staining. The membrane was scanned with the ImageQuant software.

**ES Culture and Differentiation of Embryoid Bodies**—Stem cells, from the study were the AB2.1 ES cells (21). ES cells were maintained on a layer of mitomycin D-inactivated feeders (SNL76/7). ES cells were grown on a layer of mitomycin D-inactivated mouse fibroblasts (SNL76/7). ES cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), 16% fetal bovine serum (Hyclone), 1000 units/ml leukemia inhibitory factor (Chemicon), and 5 × 10⁻⁵ M β-mercaptoethanol. Medium was changed daily, and ES cells were split every 2–3 days. We previously generated wild-type or Hmga1+/−, Hmga1+/-, and Hmga1−/− ES cells (32); Rb−/− ES cells were kindly provided (33, 34). For differentiation, ES cells were cultured as described (35). Briefly, hanging drops containing 10³ cells in 20 μl of culture medium were maintained for 2 days under the lids of bacteriological dishes filled with phosphate-buffered saline. The embryoid bodies formed were then transferred into bacteriological plates and maintained for 3 days in suspension in cultivation medium supplemented with either 0.1% DMSO or with all-trans-retinoic acid (RA) (10⁻⁸ M). Medium was changed every day. EBs were maintained 2 days more in suspension in cultivation medium and then were allowed to settle onto gelatin-coated plates in the presence of differentiation medium. This medium consists of cultivation medium supplemented with 85 nM insulin, 2 nM triiodothyronine, and 10% selected fetal calf serum (Invitrogen). Medium was changed every 2 days (36). Attached EBs were harvested after 0, 4, and 8 days for pro-
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tein analyses and after 13 days for RNA extraction. Alternatively, after 13 days, EBs were stained with Oil Red O to assess adipocyte differentiation.

**Electrophoretic Mobility Shift Assay**—Protein/DNA binding was determined by electrophoretic mobility shift assay (EMSA), as described previously (25). The E2F1 oligonucleotide (sc-2507, Santa Cruz Biotechnology) was mutated as follows (mutated bases in boldface type) in the AT-mut oligonucleotide: 5′-ACTTGGGTTCGCGCCATTCTCAA-3′. The interleukin-2 oligonucleotide was previously described (36). The DNA-protein complexes were resolved on 6% (w/v) non-denaturing acrylamide gel and visualized by exposure to autoradiographic films.

**Chromatin Immunoprecipitation and Reprecipitation**—ChIP was carried out with an acetyl-histone H3 immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer’s instruction. For ChIP experiments with the antibody anti-RB (C-15) rabbit polyclonal antibodies (Santa Cruz Biotechnology), anti-acetylated E2F1 (24), anti-HDAC1 (60-720) rabbit polyclonal antibodies (Upstate Biotechnology Inc., Lake Placid, NY), and anti-HMGA1 (polyclonal antibody raised against a synthetic peptide located in the NH2-terminal region), the conditions were as reported previously (37). For Re-ChIP experiments, complexes were first eluted by incubation for 30 min at 37 °C in 250 μl of Re-ChIP elution solution (10 mM EDTA, 40 mM Tris-HCl, pH 6.5, 40 mg/ml proteinase K) for 1 h at 37 °C. Immunoprecipitated chromatin was amplified by PCR using the following primers: Mu-cdc6-pr-up 5′-CGGCCAATTCTCGGTCA-3′; Mu-cdc6-pr-dw 5′-CTCTCGCTCCTTCCTG-3′; Mu-cdc2A-pr-up 5′-GGCTCCACCCCTCGCCTC-3′; Mu-cdc2A-pr-dw 5′-TCCTGGGTTCCCTCTCC-3′; Mu-GAPDH-pr-up 5′-TGTGAGTGAGGAGCAGC-3′; Mu-GAPDH-pr-dw 5′-TCACTGGGCTTACTGGC-3′.

**Oil Red O Staining**—After 20 days of differentiation treatment, culture medium was removed, and ES cells were washed twice with PBS. Cells were then fixed with 4% formalin/PBS. After cells were washed three times with PBS and incubated in 60% isopropyl alcohol for 10 min, the cells were stained with 1.8% Oil Red O in 60% isopropyl alcohol for 10 min. After washing three times, EBs were scored for adipocyte differentiation and photographed.

**FIGURE 1. Interaction between RB and HMGA1 proteins during adipocyte differentiation.** A, 3T3-L1 cells were treated with differentiating agents as described under “Experimental Procedures.” Cellular lysates were harvested at time 0 (0), 1 day (1d), 2 days (2d), 4 days (4d), and 6 days (6d) starting from the beginning of hormone induction. Proteins were prepared and subjected to immunoprecipitation (IP) with antibodies directed against the RB or the HMGA1 proteins, as indicated. Filters were blotted with the reciprocal antibodies. B, cellular lysates from differentiated 3T3-L1 cells were prepared at different time points. Cells were harvested at time 0 (0), 1 day (1d), 2 days (2d), 4 days (4d), and 6 days (6d) starting from the beginning of hormone induction. Levels of RB and HMGA1 during differentiation were shown. The same Western blot was reprobed with antibodies to the ubiquitous γ-tubulin protein for loading.

**RESULTS**

**Adipocyte Differentiation**—To further evaluate the interaction between HMGA1 and RB proteins during adipogenesis, we performed a pulldown assay using immunoprecipitated chromatin from adipocytes. The interaction between RB and HMGA1 was further investigated by **in vitro** and **in vivo** experiments. The cDNA encoding HMGA1b protein was inserted into GST expression plasmid, and the fusion protein GST-HMGA1b was expressed in bacteria. GST-HMGA1b fusion protein was mixed with protein extracts from RB expressing 293 cells (Fig. 2A). GST-HMGA1b (Fig. 2A, 2nd lane), but not GST alone (3rd lane), bound specifically to the hypophosphorylated isoform of RB.

For the **in vivo** experiments, 293 cells were transfected with pCMV-Rb alone or with Hmgai expression vectors. Cellular extracts were immunoprecipitated with either anti-RB or anti-HMGA1 antisera and immunoblotted with the reciprocal antibodies. Fig. 2B shows that co-expression of RB and HMGA1 proteins resulted in reciprocal co-immunoprecipitation of the two proteins. Cellular extracts derived from 293 cells used for co-immunoprecipitation assays were analyzed by Western blotting (Fig. 2C).

**HMGA1-RB Binding Is Direct**—To further evaluate the interaction between HMGA1 and RB, a pulldown assay was performed by incubating the two proteins. To this aim, full-length

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A

FIGURE 2. In vitro and in vivo interaction between RB and HMGA1 proteins. A, GST/HMGA1b (2nd lane) or the GST (3rd lane) immobilized on glutathione beads were used to bind RB in RB-overexpressing 293 cells. A control, 50 μg of RB-overexpressing 293 cell lysates was mixed with GST/GST alone (1st lane) or incubated with either anti-HMGA1 or anti-RB or control antibodies as indicated. The immunocomplexes were described in B were analyzed by Western blotting with anti-RB antibodies. As shown in Fig. 2D, 2nd lane, GST-HMGA1b was able to precipitate the RB protein. Therefore, we concluded that the two proteins interact and that the interaction is direct. The specificity of the interaction was confirmed by the observation that RB did not adhere to GST resin devoid of HMGA1b under identical conditions (Fig. 2D, 3rd lane).

C-pocket of the RB Protein Is Necessary for the Binding to HMGA1 Proteins—At least three distinct protein binding regions have been identified in RB as follows: the large A/B pocket (amino acids 395–876), which is the minimal functional domain, corresponding to the binding site for E2F heterodimers; the A/B pocket binds the LXCXE peptide motif in several proteins; the C-pocket binds the nuclear c-Abl tyrosine kinase (23). To define which region of RB protein is required for RB labeled with [35S]methionine from reticulocyte extracts was allowed to bind to glutathione S-transferase (GST-HMGA1b) immobilized on a glutathione-Sepharose matrix, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting with the anti-RB antibodies. As shown in Fig. 2D, 2nd lane, GST-HMGA1b was able to precipitate the RB protein. Therefore, we concluded that the two proteins interact and that the interaction is direct. The specificity of the interaction was confirmed by the observation that RB did not adhere to GST resin devoid of HMGA1b under identical conditions (Fig. 2D, 3rd lane).

Mapping of the HMGA1 Region Responsible for Binding to RB—To map the HMGA1 region required for binding to RB, we generated a series of progressive carboxyl-terminal deletions of the Hmgal gene (Fig. 4A). The resulting cDNAs were tagged with the influenza HA epitope into the pCEFL-HA expression plasmid and expressed in 293 cells. Cellular lysates were mixed with GST-HMGA1b in pulldown assays. The RB constructs used for this experiment are shown in the upper panel of Fig. 4D, lane 4, which shows the lowest expression plasmid encoding either wild-type or mutant RB proteins expressed in 293 cells, and then cellular lysates were probed with anti-HMGA1 or anti-RB or control antibodies as indicated. The immunocomplexes were described in B were analyzed by Western blotting with anti-RB antibodies. As shown in Fig. 4D, lane 4, wild-type RB was detected only when co-expressed with GST-HMGA1b in pulldown assays. One of the mutants used was R661W, a naturally occurring low penetrance RB allele, which contains a substitution of Arg-661 in the “B” region of RB. This mutant is defective for the LXCXE and E2F binding. The other mutant, RB 13S, has been previously generated and characterized; it contains mutations in the A/B pocket and in the C pocket. This mutant is defective for c-Abl binding (23). After the incubation, the complexes were separated by SDS-PAGE and analyzed by Western blotting with anti-RB antibodies. We determined that the carboxyl-terminal domain of RB is required for an efficient HMGA1 binding (Fig. 3B, upper panel, compare lanes 3 and 4, 13S RB and R661W RB, respectively, with lane 2, wild-type RB). A comparable level of RB expression was revealed by Western blotting with anti-RB antibodies (Fig. 3B, lower panel).

Mapping of the RB Region Required for Binding to HMGA1—To map the RB region required for binding to RB, we generated a series of progressive carboxyl-terminal deletions of the Hmgal gene (Fig. 4A). The resulting cDNAs were tagged with the influenza HA epitope into the pCEFL-HA expression vector. These mutants were tested for their interaction in vivo with RB in co-immunoprecipitation assays. Each Hmgal plasmid was transiently transfected in 293 cells together with a
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**A**

| pHA-A1b (1-96) | HA | + | + | + | ----- |
| pHA-A1b (1-79) | HA | + | + | + |
| pHA-A1b (1-63) | HA | + | + |
| pHA-A1b (1-53) | HA | + |
| pHA-A1b (1-43) | HA | |
| pHA-A1b (23-96) | HA | + | + | + | ----- |

**B**

Rb

| HA-HMGA1b (1-96) | HA | + | + | + | + |
| HA-HMGA1b (1-79) | HA | + | + | + |
| HA-HMGA1b (1-63) | HA | + | + |
| HA-HMGA1b (1-53) | HA | + |
| HA-HMGA1b (1-43) | HA | |
| HA-HMGA1b (23-96) | HA | + | + | + | + |
| HA-HMGA1b (31-96) | HA | + | + | + | + |

**C**

Western blotting showing protein expression levels of the various pCMV-Rb expressing vector. For each experiment, cells were harvested, and protein extracts were immunoprecipitated with anti-HA antibodies. Immunoprecipitated samples were then electrophoresed on a 7.5% gel and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (anti-HA) and developed with IRDye 800cw secondary antibodies. The membranes were then scanned to generate the images shown.

**Role of the Interaction between HMGA1 and RB in Adipocyte Differentiation**—In the attempt to investigate the role of the interaction between HMGA1 and RB with C/EBPβ in the activation of adipocyte-specific genes, we analyzed the activity of two promoters regulated by C/EBPβ in the presence or absence of these proteins. The obese gene codes for leptin and its expression was detected only in adipocyte cells. The Id1, Id2, and Id3 genes are negative regulators for the basic helix-loop-helix transcription factors. It has been shown that the expression of at least one of these genes, Id1, is regulated by C/EBPβ (21) and down-regulated during adipocyte differentiation (39).

First, we analyzed the ob gene promoter (−161 ob), which contains the C/EBP motif, and it is a natural target of C/EBP transcription factors (22). We showed that the ob gene promoter is activated 4–5-fold in 293 cells by C/EBPβ and 18–20-fold by the cooperative action of C/EBPβ with HMGA1 (Fig. 5A). Conversely, no activation was observed in the presence of HMGA1 alone (10), whereas we show that the presence of HMGA1 and RB together leads to a further enhancement of the ob promoter by a factor of 6–8-fold.

To evaluate the effect of HMGA1 and RB on C/EBP-mediated transcription, we used the luciferase reporter, which contains the B1 enhancer of the ob gene, and the luciferase reporter, which contains the B1 enhancer of the ob gene (Fig. 5C). Transfection of pfluc/B1s, which contains the luciferase reporter, was increased when the exogenous protein was co-expressed with the reporter (Fig. 5C).

**HMGA1 Enhances E2F-1 Activity**—Cell proliferation and differentiation have been considered to be mutually exclusive events; however, a close relationship has been established between both cellular processes during the adipocyte differentiation program (40). Re-entry into cell cycle is one of the key events taking place in early adipogenesis. As in most cells, the transition from growth-arrested adipocytes into the S-phase likely depends on the reactivation of the G1 cyclins/cyclin-dependent kinases and the retinoblastoma protein RB-E2F pathway that controls the G1/S transition of the cell cycle. Association of E2Fs with proteins of the RB family facilitates active repression through recruitment of histone deacetylases (41, 42). Upon re-entry into cell cycle of these growth-arrested adipocytes, the members of the retinoblastoma family are phosphorylated by the cyclin/cyclin-dependent kinase holoenzymes, releasing the E2F complex, resulting in the activation of the E2F target genes (43).
To better define the role of the RB/HMGA1 interaction in the regulation of E2F-1 transcriptional activity, we analyzed the activity of two E2F responsive promoter genes, such as CDC25A (44) and CDC6 (45), fused to a luciferase reporter gene, in the presence of RB and HMGA1 proteins. 3T3-L1 cells were used as recipient cells. As shown in Fig. 6,

A and C, RB represses CDC25A and CDC6 promoter activity (a reduction to 10 and 50% of the initial signal), whereas HMGA1 does not reduce the promoter activity. When RB was co-transfected with increasing levels of HMGA1, repression by RB of the CDC25A and CDC6 promoter activity was significantly antagonized by the presence of HMGA1 proteins. Western blot analysis showed that the transfected cells expressed adequate levels of the RB and HMGA1 proteins (Fig. 6, B and D).

HMGA1 Proteins Displace HDAC1 from the RB-E2F1 Complex—One of the mechanisms by which the RB-E2F complex represses transcription is the recruitment of the HDAC1 to the E2F-regulated promoters by RB (42). Histone deacetylases and histone acetyltransferases are two counteracting enzyme families whose enzymatic activity controls the acetylation state of proteins, in particular histones. Acetylation of histones regulates gene transcription through its influence on chromatin conformation. In addition, several non-histone proteins are modified in their stability or biological function by the acetylation state of their specific lysine residues.

We thus asked whether the interaction of HMGA1 with RB could displace HDAC1 from the RB-E2F complex increasing E2F1 activity as recently demonstrated for HMGA2 (19). To this aim, we performed co-immunoprecipitation assays in the 3T3-L1 preadipocytic cells transiently transfected with RB in combination or not with wild-type and mutated HMGA1 expression vectors. As shown in Fig. 7A, HMGA1 is able to reduce the binding of RB to HDAC1, although the mutant of HMGA1-(1–43), unable to bind RB, does not exert the same effect suggesting that the interaction between HMGA1 and RB proteins plays a crucial role in the displacement of HDAC1 from RB. The expression of RB, HDAC1, and HMGA1 proteins was verified by West-
ERN blotting analysis. γ-Tubulin expression was used to equalize protein loading (Fig. 7B).

To verify this result, we used a cell-free system in which RB and HDAC1 recombinant proteins were incubated with or without increasing amounts of a recombinant wild-type HMGA1b protein. As shown in Fig. 7C, HDAC1 was displaced in a dose-dependent manner by the binding to RB in the presence of 5 and 10 μg of HMGA1b recombinant protein. This result demonstrates that HMGA1b directly interferes with the binding between RB and HDAC1. In Fig. 7D, Western blot (WB) analysis shows the amount of recombinant proteins used in the co-immunoprecipitation assay shown in Fig. 7C.

HMGA1 Proteins Bind to E2F1 Target Promoters and Displace HDAC1—HMGA1 proteins allow the assembly of multiprotein complexes by directly binding to the DNA in AT-rich sequences. We used an E2F consensus oligonucleotide (E2FRE) that has an AT stretch compatible with HMGA1 binding in an EMSA with a recombinant GST-HMGA1b protein. As shown in Fig. 8A, 3rd lane, GST-HMGA1b was able to bind to the E2F-responsive oligonucleotide but not to the same oligonucleotide mutated in the AT-stretch flanking the E2F-consensus sequence (AT-mut). Binding specificity was also demonstrated by competition experiments showing a loss of binding with the addition of 200-fold molar excess of unlabeled interleukin-2 promoter region that contains AT-rich HMGA1-binding sites (36).

We next evaluated whether HMGA1 is able to bind Cdc25A and Cdc6 promoters, two E2F-responsive promoters, and whether it is able to displace HDAC1 in vivo. To this aim, we performed ChIP assays in 3T3-L1 cells transfected or not with a vector encoding HMGA1b protein. Immunoprecipitated chromatin was then analyzed by semiquantitative PCR, using primers spanning the E2F binding regions of the Cdc25A and Cdc6 promoters. Occupancy of these regions by HMGA1 was clearly
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To analyze the E2F1 acetylation status on the Cdc25A and Cdc6 promoters and transfect 3T3-L1 cells with an empty vector or a vector encoding HMGA1b protein and subjected the lysates to ChIP assay, with a specific anti-acetylated E2F1 antibody. As shown in Fig. 9A, E2F1 acetylation was enhanced by the HMGA1 overexpression. This result, consistent with our previous data demonstrating the role of the interaction of HMGA1 with RB in displacing HDAC1 from the E2F-responsive promoters, was also confirmed by an additional experiment in which total lysates from cells transfected as in Fig. 9A were analyzed by Western blot to evaluate the amount of E2F1 acetylation (Fig. 9B). Taken together, these results suggest that HMGA1 overexpression promotes the E2F1 acetylation status.

Hmga1-null ES Cells Fail to Undergo Adipocyte Differentiation and Are Required for the Induction of the RB-C/EBP Complex during Adipocyte Differentiation—To further define the role of the HMGA1 proteins in differentiation, we generated wild-type, Hmga1+/−/−, and RB−/− ES cells (32). We investigated the ability of HMGA1+/−; Hmga1−/−, and Hmga1+/− in RB+/−; ES cells to undergo adipocyte differentiation following RA treatment as described (35, 47). RB−/− ES cells failed to undergo adipogenic differentiation treatment, EBs showing a drastic reduction in the number of Oil Red O-positive EBs. This defect, reduced percentage expression was assessed with wild-type EBs, drastically reduced expression was assessed with wild-type EBs compared with DMSO-treated null clones appeared in the 3rd to 6th lanes of the semiquantitative RT-PCR panel in Fig. 10). As expected, adipocyte-specific markers by semiquantitative RT-PCR were detected in cells derived from wild-type, Hmga1+/−, and Hmga1+/−/− ES cells (32). We investigated whether the presence of the HMGA1 protein is necessary for this complex formation. First, we evaluated the induction of HMGA1, C/EBPβ, and RB expression in wild-type Hmga1+/−/−, heterozygous

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**FIGURE 7.** HMGA1 proteins compete with HDAC1 for RB binding. A, lysates from 3T3-L1 cells transfected with plasmids expressing RB in combination with or not with wild-type HMGA1b and truncated HMGA1b-(1–43) were subjected to immunoprecipitation (IP) with anti-RB antibody and then blotted with HDAC1 antibody. The right lane was used as a control of the equal loading of the immunoprecipitated RB, lysates from cells transiently transfected as in A were run on Western blot with specific antibodies to verify protein expression levels. B, IP RB was used to equalize protein loading (Fig. 8D). C/D, both recombinant proteins were co-immunoprecipitated in the presence of 5 or 10 μg of recombinant HMGA1b protein, as shown by Western blotting analysis. D, lysates were analyzed by Western blot to evaluate the amount of E2F1 expression, as assessed with either HDAC1 or RB antibody. IP,沉淀的实验；WB， Western blotting analysis.
Role of HMGA1 and RB Interaction in Adipogenesis

We then investigated whether the interaction between RB and C/EBPβ could take place in the absence of HMGA1 during adipocyte differentiation. Equal amounts of protein extracts were immunoprecipitated with anti-C/EBPβ-specific antisera. The immunocomplexes were transferred on membrane and immunoblotted with the anti-RB antibodies. The results are shown in Fig. 10C; the interaction between RB and C/EBPβ was detected only in the wild-type Hmga1+/− ES cells. Consistently, a significant reduction in the RB-C/EBPβ complex was observed in the Hmga1+/− ES lines, whereas it was completely absent in the homozygous Hmga1−/− ES cells. Moreover, we demonstrated that HMGA1-precipitated in the transfected cells, indicating that the interaction between these two proteins occur in the absence of HMGA1 (data not shown).

Expression of C/EBPβ and RB proteins (10). Therefore, the aim of this work was to investigate a possible interaction of HMGA1 with RB. Here we demonstrate that HMGA1 binds RB in 3T3-L1 cells and in 293 cells by reciprocal co-immunoprecipitation experiments. We also

FIGURE 8. HMGA1 proteins bind to E2F1 target promoters and displace HDAC1.

HMGA1 proteins bind to E2F1 target promoters and displace HDAC1. (A) Purified recombinant HMGA1 was incubated with a radiolabeled E2F-responsive oligonucleotide (E2FRE). To assess the specificity of the binding, GST-HMGA1b recombinant proteins were incubated with a radiolabeled E2F-responsive oligonucleotide (E2FRE). To assess the specificity of the binding, excess of unlabeled interleukin-2 oligonucleotide was used as competitor. (B) E2F-responsive oligonucleotide mutated in the region rich in AT bases (−) or in the presence of a 200-fold excess of unlabeled interleukin-2 oligonucleotide was used as competitor.

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Hmga1+/−, and homozygous Hmga1−/− ES cells are induced to differentiate into adipocytes. To this aim, nuclear proteins were extracted at different times of adipocyte differentiation (0 h, 4 days, and 8 days). The expression of these proteins in this system was different from that shown in the pre-adipocytic 3T3-L1 cells. In fact, HMGA1 protein levels are normally high during embryonic development and in ES cells (48). Indeed, its expression was already high at time 0, which corresponds to the beginning of the hormonal treatment, and it did not change with the treatment (Fig. 10C). C/EBPβ expression was already high, because its maximal induction is reached upon retinoic acid treatment, which precedes hormonal induction of differentiation in this system. RB protein was present in both its phosphorylated and hypophosphorylated isoforms at time 0, but it almost exclusively accumulated in its hypophosphorylated isoform following the differentiating treatment (Fig. 10C).
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Show that the GST/HMGA1b fusion protein binds RB in a typical pulldown assay. Furthermore, we showed that the binding requires the carboxyl-terminal region of RB, which is important for the inhibition of G1/S progression (23) and the NH2-terminal and the region between the second and the third AT-hooks of HMGA1b protein. HMGA1, by binding to RB and to C/EBPβ, may functionally cooperate with these proteins to activate and/or repress different promoters. In fact, HMGA1 may be involved in the assembly of highly regulated higher order complexes, which are essential both for growth arrest and for the expression of the differentiated phenotype of adipocyte precursors.

A role for the interaction of HMGA1 with RB in cell growth arrest and in modulating transcription of C/EBP-regulated genes is also confirmed by experiments regarding the transcriptional regulation of Id helix-loop-helix proteins, which act as regulatory intermediates for coordinating adipocyte differentiation. Their expression is high in proliferating cells, although it decreases in differentiating cells (21, 39). Indeed, we observed that both RB and HMGA1 proteins were able to down-regulate the activity of the Id1 promoter, and that they functionally cooperate in repression of the Id1 transcriptional activation of this promoter. The RB and RB proteins are also able to cooperate with the regulation of the obese gene, which is a natural target of C/EBP transcription factors (22).

This cooperation is able to significantly stimulate the differentiation of preadipocyte RA-induced adipocytes into adipocytes requires that growth-arrested preadipocytes differentiate and acquire the capacity to store fat (24, 25). During this phase, RA results in the coordinative expression of adipocyte differentiation commitents, which functionally cooperate with these proteins to affect the expression of their target genes (26, 27). Therefore, we investigated the role of the HMGA1 proteins in the regulation of two E2F-responsive promoter genes, such as E2F1 (44) and C/EBPβ (45), demonstrating that repression by RB of these promoters was significantly antagonized by the presence of HMGA1, which is able, such as HMGA2 (19), to displace HDAC1 from RB.

Previous studies demonstrated that retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs transcription factors. On the basis of this observation, it seemed reasonable to hypothesize a role of the HMGA1 proteins in the formation of the RB-C/EBP complex. In this study, we provide evidence supporting this hypothesis. Indeed, we were unable to detect any RB-C/EBPβ interaction in the Hmga1−/− ES cells after RA induction of the adipocyte differentiation. Conversely, the RB-C/EBPβ complex was detected in the

| FIGURE 9. HMG A1 overexpression increases acetylation of E2F1 protein. | FIGURE 10. HMGA1 is required for ES adipocyte differentiation and for the induction of the RB-C/EBP complex. |
|---|---|
| A | B |
| Cdc25A | E2F1Ac |
| Cdc6 | E2F1 |
| GAPDH | HMGA1 |
| Input | ChIP E2F1Ac |
| Tubulin | WB |
| WT | A1+/− |
| A1−/− | Rb |
| A1−/− | A1−/− |
| A1−/− | A1−/− |
| A1−/− | A1−/− |
| 0 4d 8d | 0 4d 8d |
| 0 4d 8d | 0 4d 8d |
| 0 4d 8d | 0 4d 8d |
| IP C/EBPβ | IP C/EBPβ |
| Tubulin | Tubulin |

mRNA level of Gapdh was determined by RT-PCR. The cDNAs were co-amplified with GAPDH as an internal control. Bands of comparable intensity, obtained by the mouse Gapdh sequence-specific primers, indicate comparable amplification of all samples. RNA was extracted from wild-type, Hmga1−/−, Rb−/− ES cells treated with DMSO (control) or RA and the mixture of differentiating hormones. C, levels of HMG A1, C/EBPβ, and RB in ES cells during adipocytic differentiation. 50 μg of total cell extracts from the wild-type, Hmga1−/−, Hmga1−/−, Rb−/− ES cells were separated by SDS-PAGE and transferred on Immobilon-P membranes, and filters were probed with the indicated antibodies. D, interaction between RB and C/EBPβ proteins during adipocyte differentiation of wild-type, Hmga1−/−, Hmga1−/− ES cells. Nuclear proteins were extracted from ES cells at time 0 (0), 4 days (4d), and 8 days (8d), during the induction of adipocytic differentiation, as indicated. Equal amounts of proteins were immunoprecipitated (IP) with anti-C/EBPβ antibody, and the filter was incubated with anti-RB antibody. WB, Western blot.
Role of HMGA1 and RB Interaction in Adipogenesis

retinoic acid-induced wild-type ES cells. This would suggest the requirement of HMGA1 proteins for the interaction between RB and C/EBPβ proteins. In the homozygous rb−/− ES cells, we were instead able to coprecipitate C/EBPβ and HMGA1 proteins, indicating that the interaction between these two proteins can occur also in the absence of RB. These data, taken together, indicate that HMGA1 proteins are required for the formation of the RB-C/EBPs protein complex that is essential for the expression of several adipocyte-specific genes.

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