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Biochemical and Biophysical Research Communications 364 (2007) 1050–1055
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Interaction of E2F–Rb family members with corepressors binding to the adjacent E2F site

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Received 11 October 2007
Available online 30 October 2007

Abstract

Cell cycle-dependent transcriptional repression of the *E2F1* and *B-myb* promoters is mediated through E2F-binding sites and adjacent corepressor site (cell cycle gene homology region (CHR)/downstream repression site (DRS)). Here, we show that a factor binding to the *B-myb* CHR is co-purified with E2F DNA-binding activity, and coimmunoprecipitated with components of E2F/Rb-family repressor complexes, E2F4 and retinoblastoma (Rb) family proteins. In spite of structural and functional similarities, however, the *E2F1* and *B-myb* CHRs exhibited distinct factor-binding specificities. Furthermore, substitution of *E2F1* CHR with the *B-myb* CHR in the *E2F1* promoter revealed that the *B-myb* CHR was unable to repress the *E2F1* promoter completely in the G0 phase. These results suggest that transcriptional repression of the *E2F1* and *B-myb* promoters is mediated by physical interaction of E2F/Rb-family repressor complexes with promoter-specific corepressors.

Keywords: E2F1; B-myb; Cell cycle gene homology region (CHR); Downstream repression site (DRS); Rb; Corepressor; Transcription; Repression

The E2F family of transcription factors plays a critical role in the progression of the cell cycle by regulating transcription in response to mitogenic stimulation [1,2]. The mammalian E2F family consists of nine distinct gene products that are divided into several subgroups based on structural and functional properties. In particular, E2F1, E2F2, and E2F3a act as activators of transcription, whereas E2F4 and E2F5 function primarily as transcriptional repressors in concert with members of the Rb family of tumor suppressor proteins (Rb, p107, and p130). Binding of the Rb family proteins converts E2F4 and E2F5 to active repressors of transcription. In response to mitogenic stimulation, the Rb family proteins are phosphorylated by the cyclin dependent kinases, leading to the dissociation of the E2F/Rb family repressor complexes. Previous chromatin immunoprecipitation (ChIP) studies have revealed that an E2F4/p130 complex predominantly occupies the E2F-responsive promoters in the G0 phase. Following cell cycle re-entry, the repressor complex is replaced by E2F1, E2F2 and E2F3 activators, concomitant with the induction of E2F-target gene transcription [3,4].

The *E2F1* and *B-myb* promoters are repressed in G0 and activated at the G1/S transition in an E2F-dependent manner. It has been shown that the E2F-binding sites on these promoters are required but not sufficient for the repression in G0 [5–9]. In addition to the E2F site, a CHR/DRS corepressor site, which is located adjacent to the E2F site, is required for repression [10]. The CHR was also found in the cyclin A, cdc2, cdc25C, cyclin B2, and Aurora B promoters, in which the CHR is located adjacent to a 'cell-cycle-dependent element' (CDE), and the CDE-CHR element contributes to the S/G2 phase-specific expression of these genes [11–14]. The *E2F1* promoter has two E2F sites and a CHR which is located downstream of the distal E2F side (E2F site A). The relative spacing between the CHR and E2F site A is identical to that between the

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.130

CHR/DRS and E2F site in the B-myb promoter (Fig. 1). Recently, we have shown that both the CHR and E2F site A sites are required to suppress the promoter in G0, whereas the proximal E2F site (E2F site B) contributes to transcriptional activation at the G1/S boundary [9]. In agreement with this, ChIP assays have revealed that E2F4 and p130 proteins, components of the repressor complex, binds to the E2F site A, whereas E2F1 and E2F3 activators preferentially bind to the E2F site B. Furthermore, the recruitment of E2F4 and p130 to the distal E2F site A was impaired by the mutation of the CHR site [9], which is consistent with previous observations of the B-myb promoter [8].

These observations support the notion that the cooperation between the E2F and CHR sites is requited for recruiting the E2F4/p130 repressor complex to the *E2F1* and B-myb promoters. Although a factor binding to the B-myb CHR has been detected in partially purified nuclear extracts [7], the nature of the protein(s) interacting with the CHRs remains largely unknown. Here, we show that the B-myb CHR binding activity is co-purified with E2F DNA-binding activity, and coimmunoprecipitated with E2F4 and Rb family proteins. Furthermore, we provide evidence that a factor binding to the E2F CHR is different from that of the B-myb CHR, suggesting that transcriptional repression of the E2F1 and B-myb promoters is mediated by interaction of E2F and Rb family members with promoter-specific corepressors.

Materials and methods

Cell culture. REF52 cells were cultured at 37 °C in Dulbecco's modified Eagle's minimum essential medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal calf serum (FCS). HL60 cells were maintained in RPMI 1640 medium (Nissui) supplemented with 20% FCS.

Plasmids. The human E2F1 promoter reporter constructs, E2F1-Luc (−242, wt), has been described in [5]. E2F1-Luc B-mybCHR were generated using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) with the oligonucleotides 5′-TCTTTCGCGGCAAAAAGGAAATGGC GCGTAAAAGTGGCCG-3′ and 5′-CGGCCACTTTTACGCGCCATT TCCTTTTTGCCGCGAAAGA-3′.

Transfection and luciferase assays. REF52 cells were transfected using FuGENE 6 Transfection Reagent (Roche Diagnostics) as described in [9]. A CMV β-gal vector was cotransfected as an internal control. The transfected cells were brought to quiescence in DMEM with 0.1% FCS for 48 h before harvest. Luciferase and β-galactosidase activities were measured using a luciferase reporter gene assay system (Toyo Ink) and the Aurora GAL-XE reporter gene assay system (ICN), respectively.

DNA affinity purification. Nuclear extracts were prepared from growing HL60 cells as described in [15]. To generate E2F affinity beads, four tandem repeated fragments, each of which contains either wild-type (wt) or mutant versions of two E2F sites derived from adenovirus E2 promoter (gifts from K. Ohtani), were immobilized on magnetic beads (Dynabeads M-280; Dynal). Precleared HL60 nuclear extracts (100 μg) were diluted in shift buffer (20 mM Hepes (pH 7.9), 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 0.1% Nonidet P-40, 0.2 mM DTT, 10% glycerol) buffer, containing 1.2 mg/ml bovine serum albumin, 4% ficoll, 2 mg/ml sonicated salmon sperm DNA, 1% casein,, and 1 m PMSF, and then incubated with either wt or mutant versions of the E2F affinity beads for 1 h at room temperature with gentle rotation. The beads were washed with ice-cold shift buffer containing 100 mM KCl, and the bound complexes were eluted with shift buffer containing 500 mM NaCl and 1% casein. The supernatant was dialyzed with ice-cold shift buffer.

Immunoprecipitation. Immunoprecipitation was performed as described in [15]. Briefly precleared HL60 nuclear extracts (100 μg) were incubated with antibody-conjugated Dynabeads for 4 h at 4 °C with gentle rotation. After washing, the immunoprecipitates were dissociated by treating with 0.8% sodium deoxycholate (DOC) (Sigma–Aldrich) on ice, and then the released proteins were neutralized with Nonidet P-40 (final concentration of 1%). The following antibodies were used in this study: mouse monoclonal Rb (C-36, Santa Cruz Biotechnology) and p107 (a mixture of SD-2, SD-4, SD-6, SD-9, and SD-15; gifts from N. Dyson) antibodies, and rabbit polyclonal p130 (C-18) and E2F4 (C-108) antibodies (Santa Cruz Biotechnology).

Gel mobility shift assays (EMSAs). EMSAs were performed as described in [15]. Sonicated salmon sperm DNA (500 ng) and Poly (dI–dC) (1 μg) were added as nonspecific competitors to analyze the E2F and CHR binding activities, respectively. The E2F (DHRF) probe was described previously [15]. The following double-stranded oligonucleotides were used as probes and/or unlabeled competitors: E2F wt and E2F mut; E2F1 wt and E2F1 mE2F (Mut E2F site A and B) [9]; E2F1 mCHR (5′-CGTGGCTCTTT CGCGGCAAAAAGGCCTTGGCGCGTAAAAGT-3′); E2F1 E2F1 B-myb-CHR (5′-CGTGGCTCTTTCGCGGCAAAAAGGAAATGGCGC GTAAAAGT-3′); B-myb wt (5′-TTGCCGACGCACTTGGCGGGAGA TAGGAAAGTGGTTCTGAA-3′); B-myb mE2F (5′-TTGCCGACGC ACYYGTATGGAGATAGGAAAGTGGTTCTGAA-3′); B-myb mCHR (5′-TTGCCGACGCACTTGGCGGGAGATAGGCCTGTGGTTCTGA A-3′); B-myb mE2F-mCHR (5′-TTGCCGA CGCACTTGTATGGAGA-TAGGCCTGTGGTTCTGAA-3′).

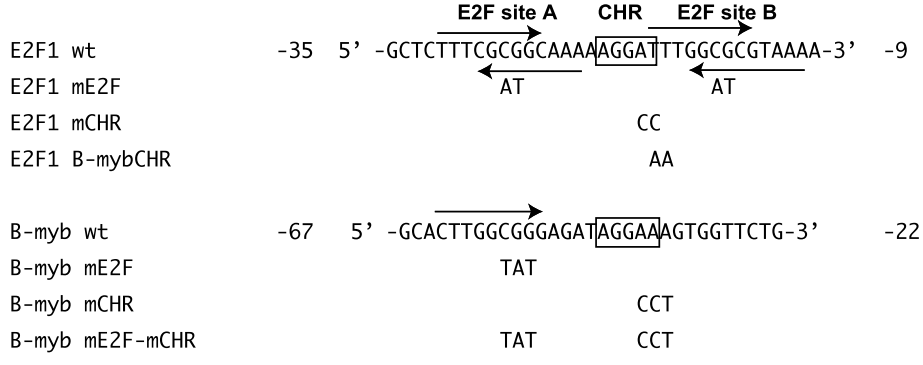

Fig. 1. Alignment of human *E2F1* and mouse *B-myb* promoters. Specific changes in these reporter constructs or the oligonucleotides used in this study are indicated below the wild-type sequences. E2F-binding sites are indicated by arrows and CHRs are boxed.