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Role for O-Glycosylation of RFP in the Interaction with Enhancer of Polycomb

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We recently demonstrated that RFP, which belongs to the large B-box RING finger protein family, interacts with Enhancer of Polycomb 1 (EPC1) and functions as a transcriptional repressor in human cultured cells. In this study, we examined the expression of RFP and EPC1 in mouse tissues by immunoblotting as well as their interaction by a pull-down assay. Both RFP and EPC1 proteins are expressed in several mouse tissues including testis, spleen, thymus, adrenal gland, cerebrum, and cerebellum. In addition, they were coprecipitated from the lysate of mouse testis. Pull-down assays using glutathione *S*-transferase (GST)-fused EPC1 proteins revealed that RFP is associated with the EPcA, EPcB, and carboxy-terminal (CT) regions of EPC1. Although RFP is highly expressed as 58- and 68-kDa proteins in mouse testis, the EPC1 CT region more strongly interacted with the 68-kDa form than the EPcA or EPcB region. Interaction of the 58-kDa form of RFP with each region was weak compared with that of the 68-kDa form with the EPC1 CT region. Because the 68-kDa form of RFP was almost completely digested with O-glycosidase but not with N-glycosidase, this suggested that O-glycosylation of RFP plays a role in its interaction with the EPC1 CT region that may be responsible for transcriptional repression. In addition, the luciferase reporter gene assay showed that expression of the EPcA region strongly impairs the transcriptional repressive activity of RFP. © 2002 Elsevier Science

Key Words: **RFP; Enhancer of Polycomb; O-glycosylation; transcriptional repression.**

RFP that belongs to the B-box RING finger protein family has a tripartite motif consisting of a RING finger, a B-box and a coiled-coil domain (1–5). We previously reported that *RFP* mRNA was detected at high levels in a variety of human and rodent tumor cell lines as well as in male germ cells (1). Immunohistochemical analysis revealed that the RFP protein is expressed in nuclei of central and peripheral neurons, hepatocytes, adrenal chromaffin cells and male germ cells (6).

Members of the B-box RING finger family are known to be involved in various cellular processes such as normal cell differentiation and growth, transformation and carcinogenesis. For example, Xnf7 is a transcriptional factor that plays a role in dorsoventral patterning during embryogenesis of *Xenopus* (7). RPT-1 has been shown to have a function in the down-regulation of interleukin 2 receptor gene and human immunodeficiency virus type 1 (8). Other members of this protein family such as RFP (1), PML (9) and TIF-1 (10) are involved in oncogenesis by the formation of fusion proteins as a result of gene rearrangement. BRCA1 (11) and ataxia–telangiectasia group D (ATDC) (12) are also related to carcinogenesis.

Polycomb group proteins have been first identified in *Drosophila* and have been shown to maintain homeotic gene repression through forming the chromatin-associated protein complexes (13–16) that could make transcription-factor binding sites inaccessible (17) or prevent enhancer-promoter interaction (18–20). Enhancer of Polycomb (EPC) is a unique member of Polycomb group proteins (13). Although mutations in *E(Pc)*, the *Drosophila* homologue of *EPC*, exhibit no homeotic transformation, they enhance homeotic mutations by other Polycomb group genes. Interestingly, a recent study showed that mutations in six Polycomb group genes including *E(Pc)* enhanced phenotypes of trithorax group mutations (21), suggesting that these genes are required not only to suppress but also to activate homeotic gene expression. Based on this finding, Brock and van Lohuizen proposed that these six Polycomb group genes are renamed the "enhancers of *trithorax* and *Polycomb*" *(ETP)* (22).

Abbreviations used: EPC, Enhancer of Polycomb; GST, glutathione *S*-transferase; NANase II, *N*-acetylneuraminidase II; PNGase F, peptide:N-glycosidase F; O-GlcNAc, O-linked *N*-acetylglucosamine.

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Using yeast two-hybrid screening, we recently found that RFP interacts with the EPC1 protein and that this interaction is mediated by binding between the coiled-coil domain of RFP and the EPcA domain or the carboxy-terminal region of EPC1 (23). In addition, we showed that RFP acts as a strong transcriptional repressor whereas EPC1 has dual functions with repressive and transactivating activities. The aim of this study is to investigate the interaction between RFP and EPC1 in mouse tissues and to clarify the characteristics of their interaction.

MATERIALS AND METHODS

Antibodies. Anti-RFP and anti-EPC1 antibodies were developed against synthetic peptides corresponding to the carboxyl-terminal 19 amino acids of human RFP and EPC1 proteins, respectively, as previously described (23). The antibodies were affinity-purified with the synthetic peptides.

Western blotting. Total cell lysates were prepared from mouse tissues (6- to 8-week-old) and a 293 human embryo kidney cell line. Tissues or cultured cells were lysed in sodium dodecyl sulfate (SDS) sample buffer [20 mM Tris–HCl (pH 6.8), 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg/ml bromophenol blue (BPB), 80 mM dithiothreitol (DTT)]. The lysates were boiled for 3 min. Equal protein amounts of the lysates were subjected to SDS–8% polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked for 30 min at room temperature in 3% albumin in TPBS (phosphate-buffered saline containing 0.05% Tween 20) with gentle shaking and incubated with primary antibodies overnight at 4°C. After washing the membranes three times with TPBS, they were incubated with the secondary antibody conjugated to horseradish peroxidase (swine anti-rabbit IgG-HRP, Dako, Denmark) for 1 h at room temperature. The reaction was examined by an enhanced chemiluminescence detection kit (ECL, Amersham–Pharmacia Biotech, Buckinghamshire, UK) according to the directions of the supplier.

Immunoprecipitation. Mouse testis tissue was lysed in radioimmunoprecipitation assay (RIPA) buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100] containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the protease inhibitors (1 μg/ml leupeptine, 1 μg/ml aprotinin, 5 μg/ml benzamidine, and 1 μg/ml pepstatin). The lysate was centrifuged at 15,000*g* at 4°C for 20 min. The protein concentration of the supernatant was measured using the BCA protein assay kit (Pierce, Rockford, IL). The lysate was preincubated with protein A–Sepharose beads (Sigma, St. Louis, MO), and then centrifuged at 5000*g* for 5 min to remove the beads. The supernatant was incubated with 2 μg of anti-RFP antibody or anti-EPC1 antibody for 1 h at 4°C, and then mixed with protein A–Sepharose beads. The mixture was incubated for 1 h at 4°C and centrifuged at 5000*g* at 4°C for 1 min. After washing three times in lysis buffer and twice in high salt buffer, the sample was suspended in SDS–sample buffer and boiled for 5 min. Then the protein A–Sepharose beads were removed by centrifugation, and the proteins were separated by SDS–8% polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore). The membranes were analyzed by immunoblotting with anti-RFP or anti-EPC1 polyclonal antibodies.

Plasmid construction and generation of GST-fusion proteins. Four *EPC1* cDNA fragments containing EPcA domain region (A region, amino acids 2 to 285), EPcB domain region (B region, amino acids 280 to 496), EPcC domain and glutamine-rich region (CQ region, amino acids 493 to 620), or CQ and carboxyl-terminal region (CQCT region, amino acids 493 to 836) (Fig. 2A) were amplified by the specific primers with a flanking *Eco*RI site on the 5′ primer and an *Xho*I site on the 3′ primer, using the full-length *EPC1* cDNA (23) as a template. A cDNA sequence corresponding to each EPC1 domain was inserted into the *Eco*RI/*Xho*I sites of pGEX-KG plasmid (Amersham–Pharmacia Biotech). The resulting recombinant plasmids were used to transform *Escherichia coli,* DH5α. Bacteria cultures were grown in Luria Bertonia medium containing 50 μg/ml ampicillin and induced with 0.2 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 2 h until they reached 0.5–0.8 at OD_{600}. The induced GST–EPC1 fusion proteins were purified with glutathione–Sepharose 4B beads (Amersham–Pharmacia Biotech) and eluted by glutathione. After eliminating glutathione by dialysis in ice-cold PBS buffer, the concentration of each GST-fusion protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Pull-down assay. Mouse testis was lysed in RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA (pH 8.0) and 0.5 mM sodium orthovanadate] containing 1 mM PMSF and the protease inhibitors (leupeptine, aprotinin, benzamidine, and pepstatin). The cell lysate was centrifuged at 15,000*g* at 4°C for 20 min to remove the cell pellet. The protein concentration of the supernatant was measured using the BCA protein assay kit (Pierce). The supernatant was precleared with GST and glutathione agarose beads for 30 min at 4°C, and then centrifuged at 5000*g* for 5 min to remove the beads. The lysate was incubated with 5 μg of GST-fusion proteins or GST plus glutathione beads at 4°C for 2 h. The glutathione beads were washed with RIPA buffer three times. The proteins bound to GST–EPC1 fusion proteins were eluted in SDS-sample buffer, subjected to SDS–PAGE and immunoblotted with anti-RFP antibody as described above.

Digestion of RFP with glycosidases. A total cell lysate was prepared from mouse testis in which 58- and 68-kDa forms of RFP are expressed at high levels. In this experiment, *N*-acetylneuraminidase II (NANase II), O-glycosidase DS and Peptide:N-glycosidase F (PNGase F) were used for digestion of α(2–3) and α(2–6)*N*-acetylneuraminic acids, sialylated and non-sialylated O-linked core structures of Gal(β-1,3)GalNAc(α1), and Asn-linked oligosaccharides, respectively. For digestion with N- and O-glycosidases, 12 μl of immunoprecipitated RFP protein solution was used according to the manufacturer's protocol (enzymatic deglycosylation kit, Bio-Rad). Briefly, after adding 4 μl of 5× reaction buffer (250 mM sodium phosphate, pH 6.0), the sample was incubated with 2 μl of NANase II (5 U/ml) for 1 h at 37°C. Then, 2 μl of O-glycosidase DS (1 U/ml) was added and the sample was incubated at 37°C overnight. After incubation, 10 μl of deionized water, 10 μl of pH adjustment buffer (0.5 M sodium phosphate dibasic) and 2.5 μl of denaturation solution (2% SDS, 1 M β-mercaptoethanol) were added to the vial. The reaction vial was then heated for 5 min at 100°C and cooled on ice for 5 min, followed by the addition of 2.5 μl of NP-40. After mixing the solution, the sample was incubated with 2 μl of PNGase F (2.5 U/ml) for 3h at 37°C. It was subjected to SDS–8% polyacrylamide gel electrophoresis, followed by immunoblotting with anti-RFP antibody. The addition of O-glycosidase DS and NANase II or PNGase F was skipped for digestion of N-glycosylation or O-glycosylation only.

Luciferase reporter gene assay. Constructs for the reporter assay were described previously (23). Each domain region of *EPC1* cDNA was inserted into the pFLAG-CMV2 expression vector. 293 human embryo kidney cells were cultured in 24-well tissue culture plates and cotransfected with 20 ng of luciferase reporter plasmid, 20 ng of pRL-TK plasmid (Promega, Madison, WI), 80 ng of pCMV GAL4-RFP and 300 ng of pFLAG-CMV2 EPC1 constructs by LipofectAMINE PLUS method (Invitrogen, Carlsbad, CA). The cells were harvested 48 h after transfection, and luciferase assays were performed as described previously (23). Cotransfection with the pRL-TK plasmid was used to normalize all luciferase values.

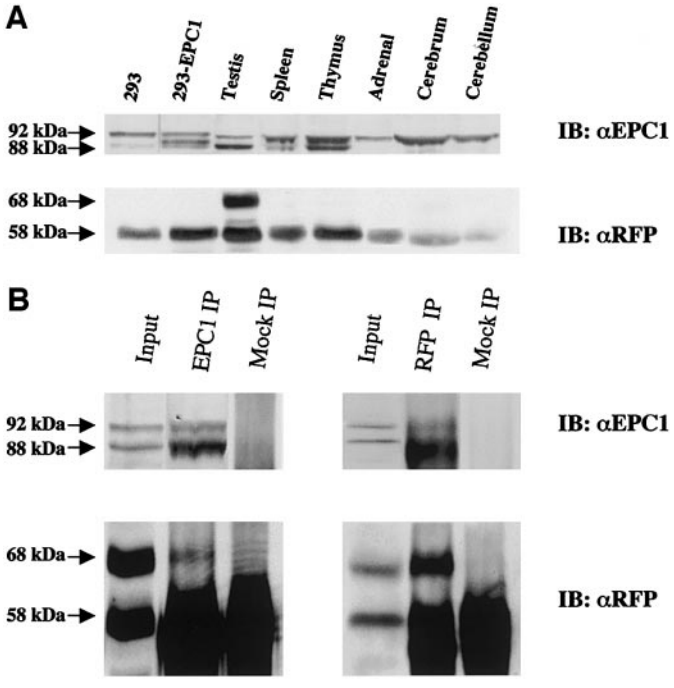

FIG. 1. Expression of EPC1 and RFP and their association in mouse tissues. (A) Total cell lysates (20 μg of proteins) were analyzed by immunoblotting with anti-EPC1 (top) or anti-RFP antibody (bottom). We used the lysate from a 293 human embryo kidney cell line as a positive control for detection of the EPC1 proteins (23). 293-EPC1 is a 293 cell line transfected with FLAG-tagged human *EPC1* cDNA (23). (B) The lysates from mouse tissues were immunoprecipitated with anti-EPC1 or normal rabbit IgG (Mock), followed by immunoblotting with-anti EPC1 or anti-RFP antibody (left). Reciprocally, the lysate was immunoprecipitated with anti-RFP antibody, followed by immunoblotting with anti-EPC1 or anti-RFP antibody (right). IP, immunoprecipitation; IB, immunoblotting.

RESULTS

First, we examined the expression of RFP and EPC1 proteins in mouse tissues by immunoblotting. As previously reported, RFP is expressed as 58- and 68-kDa proteins in mouse testis and mainly as 58-kDa proteins in other tissues including spleen, thymus, adrenal gland, cerebrum and cerebellum (Fig. 1A). A low level of expression of 68-kDa form was also detected in the latter tissues when a longer exposure of the blotting film was carried out (data not shown). EPC1 was expressed as 92- and 88-kDa proteins in mouse tissues examined although the expression level of each form was variable among them (Fig. 1A).

To elucidate the interaction of EPC1 with RFP in mouse tissues, the lysate from mouse testis was immunoprecipitated with anti-EPC1 antibody, followed by immunoblotting with anti-RFP antibody. As shown in Fig. 1B, the 68-kDa form of RFP was coprecipitated with the EPC1 protein. Detection of the 58-kDa form of RFP in coprecipitation was impossible in this experiment because this band was superimposed by that of anti-EPC1 antibody used for immunoprecipitation (Fig. 1B). Reciprocally, when the lysate was immunoprecipitated with anti-RFP antibody, followed by immunoblotting with anti-EPC1 antibody, coprecipitation of EPC1 with RFP was also found, indicating the association of these two proteins in mouse testis.

The EPC1 protein contains EPcA, EPcB, EPcC domains and a glutamine-rich (Qx) region (Fig. 2A) that are conserved in many species (23). Using yeast two-hybrid assay, we previously demonstrated that the EPcA domain and the carboxy-terminal region of EPC1 were associated with RFP (23). To further investigate the binding ability of each domain of EPC1 with RFP, we produced GST–EPC1 fusion proteins (Fig. 2B) and performed the pull-down assay. The GST-fusion proteins including each domain of EPC1 were incubated with the lysate of mouse testis and the bound proteins were analyzed by immunoblotting with anti-RFP antibody. As shown in Fig. 2C, GST–EPC1(CQCT) fusion proteins containing the carboxy-terminal region strongly interacted with the 68-kDa form of RFP, whereas the interaction of this fusion protein with the 58-kDa form was weak. In addition, the GST–EPC1(A) and GST–EPC1(B) fusion proteins weakly interacted with both forms of RFP (Fig. 2C).

As described above, the 68-kDa form of RFP is highly expressed in testis whereas its expression is very low in other tissues. Although we isolated and sequenced more than 10 *Rfp* cDNA clones from mouse testis cDNA library, we identified a single open reading frame in all of them that was identical to the previously reported *Rfp* sequence (24). Thus, the difference between 68 kDa and 58 kDa forms could be due to post-translational modification of the protein. To investigate whether these two forms show the difference of glycosylation, the cell lysate from mouse testis was digested with Peptide:N-glycosidase F (PNGase F) or O-glycosidase DS and *N*-acetylneuraminidase II (NANase II). As shown in Fig. 3, when digested with O-Glycosidase DS and NANase II (lanes 4 and 5), the band corresponding to the 68 kDa form almost disappeared, suggesting that O-glycosylation in the 68 kDa RFP form plays a role in the strong interaction with the carboxy-terminal region of EPC1. In contrast, digestion with N-glycosidase alone did not significantly change the amounts of both forms (lane 3) compared with those in the control (lane 2).

We previously reported that RFP strongly represses the gene transcription activity independent of the differences of enhancers and promoters used (23). Thus, we further investigated the effect of each region of EPC1 on the repressive activity of RFP. We made a luciferase reporter plasmid containing five tandem repeats of GAL4 binding site followed by serum-responsive element (SRE) as an enhancer and SV40 promoter (Fig. 4A). The effector plasmid was constructed by ligating GAL4-binding domain (GAL4BD)

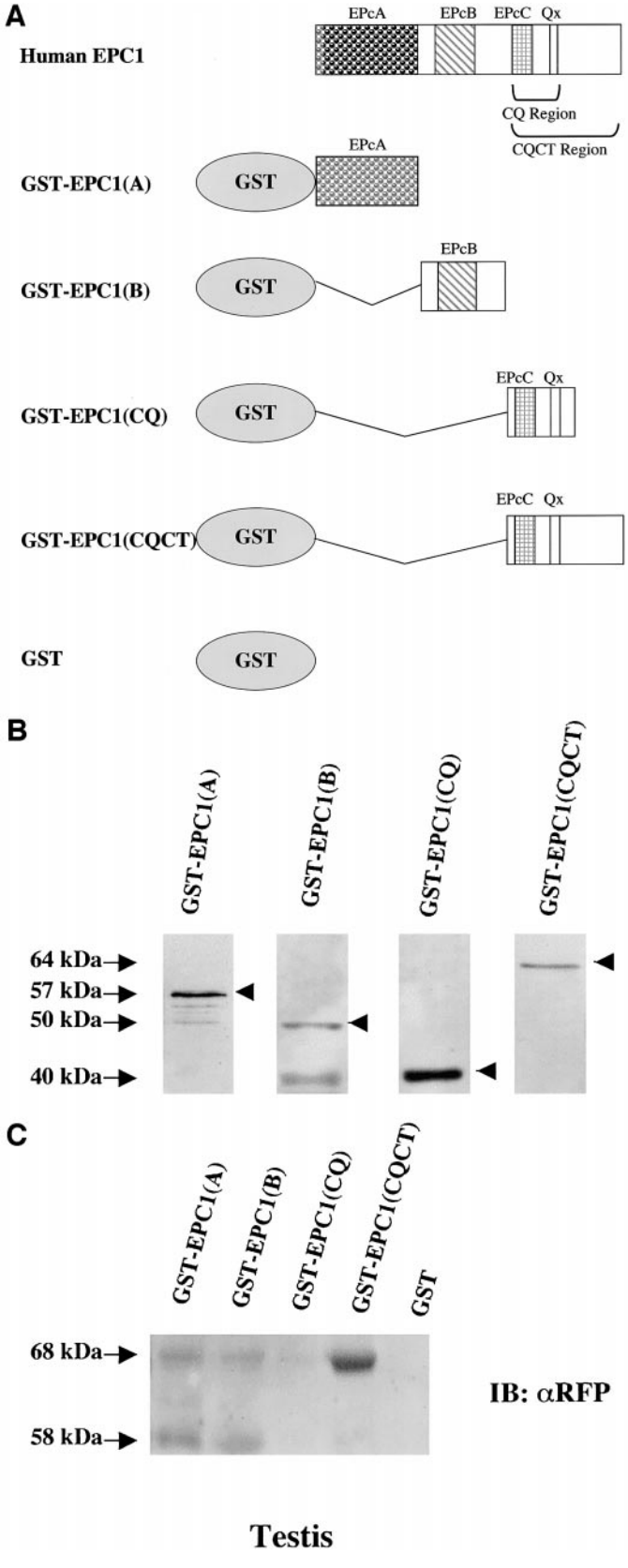

FIG. 2. Pull-down assay with GST–EPC1 fusion proteins. (A) A schematic illustration of GST–EPC1 fusion proteins produced. (B) Purified GST-fusion proteins are shown. Arrowheads indicate the expected bands of the fusion proteins containing the designated EPC1 domain regions. (C) A total cell lysate from testis was pull-downed with the designated GST–EPC1 fusion proteins, and bound proteins were analyzed by immunoblotting with anti-RFP antibody.

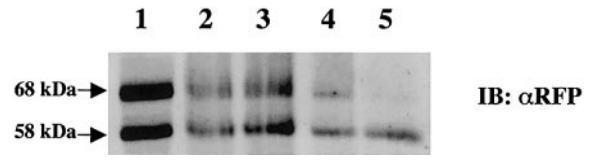

FIG. 3. Digestion of RFP proteins with glycosidases. The lysate from mouse testis was immunoprecipitated with anti-RFP antibody and incubated in the absence (lane 2) or presence (lanes 3 to 5) of the following enzymes. Lane 3, Peptide:N-glycosidase F (PNGase F); lane 4, PNGase F, *N*-acetylneuraminidase II (NANase II) and O-glycosidase DS; lane 5, NANase II and O-glycosidase DS. The resulting samples were analyzed by immunoblotting with anti-RFP antibody. The total cell lysate from mouse testis was used to show the location of 68- and 58-kDa forms of RFP (lane 1).

in-frame with full-length *RFP* cDNA (Fig. 4A). Expression of GAL4-RFP construct repressed the luciferase activity by approximately 80%, compared with expression of the GAL4BD only (Fig. 4B). This repressive activity of RFP was markedly impaired by cotransfection with the expression plasmid containing the EPC1(A) region whereas cotransfection with the expression plasmid containing the EPC1(B) region, the EPC1(CQCT) region or full-length EPC1 did not significantly affect its repressive activity (Fig. 4B). This finding suggested that each region of EPC1 differently regulates transcriptional repressive activity of RFP.

DISCUSSION

Using yeast two-hybrid system, we recently identified EPC1 as an interacting protein of RFP in human cultured cells (23). In this study, we found that both RFP and EPC1 are expressed in several mouse tissues including testis, spleen, thymus, adrenal gland, cerebrum and cerebellum, and confirmed the association between RFP and EPC1 in mouse testis in which RFP is highly expressed. EPC1 contains four domains conserved among species including EPcA, EPcB and EPcC domains and a glutamine-rich region. To further characterize the binding ability of each domain of EPC1 to RFP, we investigated it by a pull-down assay using the GST–EPC1 fusion proteins. As a result, we found that the carboxy-terminal region of EPC1 is strongly associated with the 68 kDa form of RFP that is highly expressed in testis whereas the binding ability of its 58-kDa form to this region is weak, suggesting a functional difference between the 58- and 68-kDa forms of RFP. In addition, both forms weakly interacted with the EPcA and EPcB domain regions.

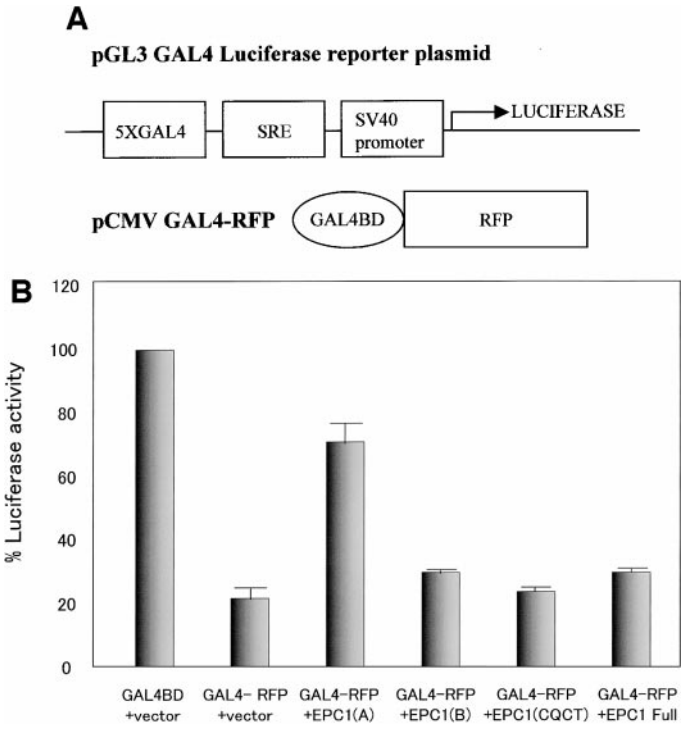

FIG. 4. Effects of different regions of EPC1 on transcriptional repressive activity of RFP. (A) Physical map of reporter and effector constructs. The GL3 luciferase reporter plasmid (Promega) contains five tandem repeats of GAL4-binding sites (5× GAL4), followed by serum responsive element (SRE) as an enhancer and SV40 promoter. The effector plasmid was constructed by ligating GAL4-binding domain (GAL4BD) in-frame with full-length *RFP* cDNA. (B) Impairment of transcriptional repressive activity of RFP by the EPcA domain region. The reporter plasmid, the pCMV GAL4-RFP effector plasmid and the pFLAG-CMV2 expression plasmid with or without each domain region of EPC1 were cotransfected into 293 cells. Luciferase activity in cells transfected with the plasmid containing GAL4BD alone was set at 100% and luciferase activities of cells transfected with the designated effector and expression plasmids were expressed as percentages of control value. Each value represents a result of at least three experiments and bars indicate standard errors.

Because we were able to detect no difference of coding sequences among more than 10 *Rfp* cDNA clones isolated from mouse testis cDNA library, we asked if the difference between 58- and 68-kDa forms is due to post-translational glycosylation. Interestingly, our results demonstrated that the 68 kDa form was almost completely digested with O-glycosidase DS and NANase-II whereas the molecular mass of the 58-kDa form was not affected. It is well known that a variety of nuclear and cytosolic proteins are glycosylated at specific serine or threonine residues by O-linked *β-N*-acetylglucosamine (O-GlcNAc) (25). These include RNA polymerase II, numerous transcription factors, chromatin associated proteins, protooncogenes and tumor suppressors (26). O-linked glycosylation of nuclear proteins such as Sp1 appears to give rise to functionally distinct subsets of the proteins. Jackson and Tjian reported that O-glycosylation of Sp1 may regulate directly its transcriptional activation and DNA binding (27). In addition, it was shown that the presence of O-glycosylation inhibited both Sp1 dimerization and interaction of Sp1 with TATA binding protein-associated factor 110 *in vitro* (28).

Another well known example is the c-Myc oncoprotein which contains multiple O-GlcNAc residues in the transactivation domain (29). The transactivation domain of c-Myc was associated with the Rb protein and the Rb-related proteins p107 *in vitro*. It was suggested that alterations of the glycosylation site in the transactivation domain impair the normal Rb-mediated control of transactivation of c-Myc and result in an aberrantly activated oncoprotein (26). The exact functional significance of O-glycosylation in the 68-kDa form of RFP is currently unknown. However, based on our findings, it could be speculated that O-glycosylation of RFP may play a role in protein-protein interactions (namely with EPC1) and thus in the regulation of transcription.

The luciferase reporter gene assay revealed that expression of the EPcA domain region markedly impaired the transcriptional repressive activity of RFP. Taken together with a recent study showing that mutations in *Drosophila E(Pc)* enhanced phenotypes of trithorax group mutations (21), our finding suggests that the EPcA domain may be involved in the activation of homeotic gene expression. In contrast, the expression of EPcB domain or CQCT region of EPC did not significantly affect the repressive activity of RFP. Although the CQCT region of EPC did not enhance its repressive activity, this may be due to sufficient expression of endogenous EPC proteins in cultured cells. Thus, the fact that the 68-kDa O-glycosylated form of RFP strongly interacted with the CQCT region of EPC suggested that this interaction could be important to maintain the repressive activity of RFP in testis that may be involved in germ cell differentiation.

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