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Expression of a transactivation-deficient form of thyroid transcription factor I decreases the activity of co-transfected thyroglobulin and thyroperoxidase promoters

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Abstract Thyroid transcription factor I (TTF-1) plays a critical role in thyroid organogenesis and in the control of expression of several thyroid-specific genes, like those coding for thyroglobulin and thyroperoxidase. We have expressed the isolated DNA-binding homeodomain of TTF-1 in cultured thyroid cells by transient transfection. A specific reduction in the activity of co-transfected thyroglobulin and thyroperoxidase promoters was observed in the presence of the isolated TTF-1 homeodomain, as compared to their activity measured in the presence of a mutated homeodomain unable to bind DNA. The activity of the SV40 early promoter, used as a control, was only marginally affected in these experiments. The transactivation-deficient form of TTF-1 described here may thus be used for investigating other cellular processes that are dependent on TTF-1 transcriptional activity.

Key words: TTF-1; Homeodomain; Thyroglobulin; Thyroperoxidase; Transcription; Thyroid

1. Introduction

Thyroid transcription factor I (TTF-1) is a homeodomain-containing protein that was identified first in the thyroid and was shown to present a restricted tissue distribution [1,2]. It controls the expression of several thyroid-specific (thyroglobulin, thyroperoxidase, TSH receptor) [3] and pulmonary-specific (surfactant proteins A, B and C) genes [4–6]. Targeting of the TTF-1 gene in mice resulted in a lethal phenotype with defects in the organogenesis of the thyroid, lung, ventral forebrain and pituitary [7]. In vitro studies using TTF-1 antisense oligonucleotides also indicated that this factor plays a role in the control of thyroid cell proliferation [8] and lung branching morphogenesis [9].

The investigation of structure/function relationships in the TTF-1 molecule revealed that the central homeodomain is flanked on both sides by transcriptional activation regions [10]. The isolated homeodomain itself, produced in bacteria, exhibited all the DNA-binding properties of the entire protein as assayed in vitro [11]. On the basis of these data, it was tempting to speculate that the isolated TTF-1 homeodomain would behave as a dominant negative form of TTF-1 when overexpressed in the cell. We have generated an expression construct that directs the production of the isolated TTF-1 homeodomain in eukaryotic cells and we show here that the expressed protein is able to repress TTF-1 transcriptional activity in transfected cells.

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2. Materials and methods

2.1. DNA constructions

Cloning experiments were performed according to standard procedures [12]. Plasmid bTgCAT6 was constructed by transferring the *Hin*dIII-*Sac*I subfragment from plasmid TgCAT14M [13] that contains the bovine thyroglobulin promoter region (from −172 to +9) fused to the CAT coding sequence into the pBLCAT6 vector [14]. Plasmid bENHbTgCAT6 was obtained by inserting the upstream enhancer element from the bovine thyroglobulin gene (−1906 to −1744) [15] into *Hin*dIII+*Bam*HI cleaved bTgCAT6. To construct the expression plasmid HD, the sequences encoding the homeodomain of dog TTF-1 [16] were amplified using the following primers: 5′ primer (64-mer): GGCGAATTCCGCCATGGCACCAAAAAAGAAGAGAA-AGGTGGAGGCCCCGCTGCCAAGCGCACCG; 3′ primer (29-mer): GGCGGATCCTACTGCGCCGCCTTGTCCTT. The 5′ primer contains a translation initiation ATG codon followed by the sequences coding for the nuclear localization signal from SV40 large T antigen [17]. The amplified fragment was cloned into *Eco*RI+*Bam*HI cleaved pSG5 expression vector (Stratagene, La Jolla, CA, USA) and sequenced in order to exclude the presence of PCR-introduced mutations. The construction of the HDm plasmid involved the mutagenesis of the sequences coding for residues 48–50 in the TTF-1 homeodomain in a first PCR reaction using the following 5′ primer (33-mer; introduces a *Not*I restriction site in the sequence): CAGGT-CAAAATCGCGGCCGCGAACCACCGCTAC, and the same 3′ primer as used above. The 80 bp PCR product was purified, treated with T4 DNA polymerase in the presence of the four dNTPs and used as a 3′ mega-primer [18] in a second PCR reaction (5′ primer: 64 base primer used for HD construction). The amplified fragments were cloned in pSG5 and clones containing the desired mutation were selected on the basis of the presence of the expected *Not*I restriction site.

2.2. Cell culture, DNA transfections and CAT assays

FRTL5 cells were maintained in F12-Coon's medium supplemented with 5% fetal calf serum, 5 μg/ml transferrin, 1 μg/ml insulin, 1 mU/ml TSH, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. Co-transfections were achieved using the calcium-phosphate technique [19] and a 1:1 ratio of expression and reporter constructs. Total amounts of DNA engaged in the transfection varied from 4 to 8 μg per 60 mm diameter culture dish. CAT assays were performed as described [15]. The background value represented less than 10% of the lowest value measured in the assay.

3. Results and discussion

We decided to investigate the effect of the production in the cell of the isolated TTF-1 homeodomain on the activity of transfected promoters known to be transactivated by the endogenous TTF-1. In order to ensure proper nuclear targeting of the produced protein, the sequences coding for the TTF-1 homeodomain were fused in 3′ of the sequences encoding the nuclear localization signal of the SV40 large T antigen using the PCR technique. The resulting DNA fragment was cloned into the pSG5 vector (Stratagene), giving the expression construct 'HD'. As we needed an appropriate control in the co-

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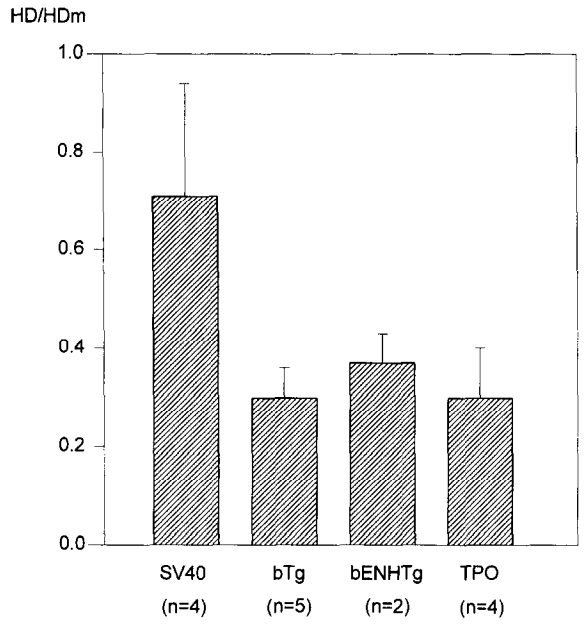

Fig. 1. Results of the co-transfection experiments in FRTL-5 cells expressed as the ratio of CAT activity measured in the presence of the wild-type homeodomain (HD) to that measured in the presence of the mutated homeodomain (HDm) for each reporter construct (see also text; the number of independent transfection experiments done appears in parentheses).

transfection experiments, a second expression construct, 'HDm', was also generated. This construct specifies the production of a mutated homeodomain in which residues 48–50 (Trp-Phe-Gln, in helix III of the homeodomain) are replaced by alanines. This mutation is known to result in a null DNA-binding phenotype [20].

The reporter constructs contained the chloramphenicol acetyltransferase (CAT) gene under the control of thyroid-specific promoter elements from the thyroglobulin and thyroperoxidase genes. Construct bTgCAT6 (bTg in Fig. 1) harbors the proximal promoter region from the bovine thyroglobulin gene [13], and construct bENHbTgCAT6 (bENHTg in Fig. 1) contains in addition the upstream element from the same gene [15]. In construct pTPOCAT (TPO in Fig. 1), the CAT gene is under the control of the human thyroperoxidase promoter [21]. All of these regulatory regions have been shown to be functionally dependent on TTF-1 in transfection experiments [3]. The pSV2CAT plasmid [19] (SV40 in Fig. 1) was used as a control reporter construct, as there are at present no indications that the activity of the SV40 early promoter depends on the presence of transcriptionally competent TTF-1.

The co-transfection experiments were conducted in the thyroid cell line FRTL-5. These cells express the endogenous TTF-1 gene and contain the other factors required for the activation of the thyroid-specific promoters. Each reporter construct was co-transfected with the HD and the HDm expression plasmids in separate dishes, in duplicate. CAT activities were measured and results were expressed as the ratio of CAT production obtained in the presence of HD (wild-type homeodomain) to that obtained in the presence of HDm (mutated homeodomain). As is apparent in Fig. 1, when the reporter construct harbored a TTF-1-dependent promoter (bTg, bENHTg and TPO), this ratio was significantly lower than that measured for pSV2CAT (SV40). This demonstrated that the expressed TTF-1 homeodomain, when able to bind DNA, could compete out transactivation by the endogenous factor. However, we noted that no complete suppression of the activity of the reporter promoters was obtained and, concomitantly, that a slight nonspecific decrease in the activity of the SV40 promoter was observed in most experiments. The extent of this nonspecific effect was related to the extent of repression on the activity of the thyroid-specific promoters in all experiments. Extreme experimental results (expressed in the form of HD/HDm values as in Fig. 1) were as follows: experiment 1: bTg: 0.4 ± 0.05 and SV40: 1 ± 0.15 (no nonspecific effect); experiment 2: bTg: 0.22 ± 0.04 and SV40: 0.36 ± 0.03 (large nonspecific effect but still significant specific effect). No correlation was found between the amount of DNA (total of 4–8 μg per dish; a 1:1 ratio of expression to reporter construct was maintained) engaged in the transfection and the extent of repression (both specific and nonspecific). As the results of duplicates within a given experiment were consistent, it most likely resulted from differences in transfection efficiencies between the individual transfection experiments. It is conceivable that a higher transfection efficiency will result in an overall increase in the production of the transactivation-deficient form, which would in turn entail a more pronounced repression. At high titers, DNA binding by the TTF-1 homeodomain would then also affect transcriptional activity in a nonspecific way. This view is supported by the data recently reported by Fabbro et al. [22] indicating that the panel of sequences recognized by the TTF-1 homeodomain is wider than expected previously.

This difficulty requires that appropriate experimental conditions should be defined, and appropriate controls should be used, in each application in order to achieve a compromise allowing the clear detection of the specific effects. As shown here, provided adequate conditions are found, the expression of the transactivation-deficient form of TTF-1 described may specifically interfere with TTF-1 action. This ability could be used to characterize other cellular processes relying on the transactivation potential of TTF-1.

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