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Calreticulin Enhances the Transcriptional Activity of Thyroid Transcription Factor-1 by Binding to Its Homeodomain*

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Transcription factors are often regulated by associated protein cofactors that are able to modify their activity by several different mechanisms. In this study we show that calreticulin, a Ca^{2+}-binding protein with chaperone activity, binds to thyroid transcription factor-1 (TTF-1), a homeodomain-containing protein implicated in the differentiation of lung and thyroid. The interaction between calreticulin and TTF-1 appears to have functional significance because it results in increased transcriptional stimulation of TTF-1-dependent promoters. Calreticulin binds to the TTF-1 homeodomain and promotes its folding, suggesting that the mechanism involved in stimulation of transcriptional activity is an increase of the steady-state concentration of active TTF-1 protein in the cell. We also demonstrate that calreticulin mRNA levels in thyroid cells are under strict control by the thyroid-stimulating hormone, thus implicating calreticulin in the modulation of thyroid gene expression by thyroid-stimulating hormone.

Thyroid transcription factor-1 (TTF-1)[1] is a homeodomain-containing protein implicated in the transcriptional activation of genes expressed exclusively in thyroid (1, 2) and lung (3). In addition, TTF-1 plays an important role in thyroid, lung, and brain morphogenesis before the onset of cell type-specific transcription (4). The ability of TTF-1 to activate transcription of different genes in distinct cell types and to play diverse roles during development suggests that the activity of this transcription factor is highly regulated.

Mechanisms involved in modulating the transcriptional potential of a transcription factor, without changes in its intracellular concentration, are post-translational modification, such as phosphorylation (5), and/or modulation of the redox state (6). Cofactors have also been shown to be important modulators of transcription factors activity and, in some cases, to be able to assign to a given transcription factor distinct promoter specificity in defined cell types (7–10). Cofactors are important for transcriptional activation through different mechanisms; they can act as a bridge between a transcription factor and the basal transcription apparatus (11, 12), they can be involved in chromatin reorganization (13), or they can modify the DNA binding properties of a transcription factor (14–16). Other proteins act as cofactors by activating the conformation of transcription factors and modulating in this way their DNA binding capability (17).

The DNA binding activity of TTF-1 has been proposed to be regulated by redox (18, 19) and phosphorylation (20–23), even though for the latter modification no effect has been observed in heterologous cells (24). However, no TTF-1 cofactor has been identified yet.

We searched for TTF-1 cofactors using a modified yeast one-hybrid system and found that TTF-1 can interact with calreticulin, a 60-kDa protein and a major Ca^{2+}-binding component in non-muscle cells. Calreticulin plays a role in Ca^{2+} storage, its expression is modulated during cell differentiation, and the amount of this protein varies among different cell types (25). Calreticulin is also an important chaperone involved in glycoprotein maturation (26) as it promotes the efficient folding and assembly of class I histocompatibility molecules (27). Moreover, it has been demonstrated that calreticulin modulates gene expression by interaction with steroid hormone nuclear receptors (15, 16).

We show in this report that TTF-1 and calreticulin interact both *in vitro* and *in vivo*. Overexpression of calreticulin in HeLa cells results in an increased activity of TTF-1, suggesting that the interaction between these two proteins may have functional significance. Calreticulin binds to the TTF-1 homeodomain and may act by promoting its folding because the DNA binding activity of heat-denatured TTF-1 HD synthesized in *Escherichia coli* is improved significantly by calreticulin. These findings suggest that calreticulin is able to modulate TTF-1 activity by regulating the folding state of TTF-1 HD.

EXPERIMENTAL PROCEDURES

Plasmids—The reporter plasmid Tg-βGal has been described (28). The TTF-1 deletion mutant Δ36 (29), containing the TTF-1 coding sequence from amino acid 96 to 296, was inserted downstream of the ADH1 promoter in the plasmid pGAD424 (CLONTECH), replacing the GAL4 activation domain. From this plasmid we obtained, by cleavage with *Sph*I, a fragment containing the ADH1 promoter, Δ36, and ADH1 terminator sequence. Cloning this fragment in pGBT9 plasmid (CLONTECH) by replacing the fragment between the *Sph*I sites yielded the plasmid ADH1Δ36. The plasmids Tg-βGal and ADH1Δ36 were used for the yeast one-hybrid system. The complete sequence of the calreticulin open reading frame was cloned by reverse transcriptase-polymerase chain reaction using RNA extracted from the rat thyroid cell FRTL-5 (29). The plasmid CMVcalreticulin was generated by inserting the entire calreticulin cistron in the expression vector pKW10 (30). The plasmids CMVTTF-1, ΔG7, ΔG13, G5E1b, C5E1b, CMV-Luc, and pTA-CAT3, used in transient transfection of HeLa cells, have been described previously (29, 31).

Yeast Strains and Methods—The yeast strain used was INVSC1 (MATa, his3-Δ1, leu2, trp1–289, ura3–52) (Invitrogen). Yeast were grown in YEPD or selective minimal medium (32). Transformations were made by the method of Schiestl and Gietz (33). β-Galactosidase activity was assayed in liquid as described previously (34).

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[1] The abbreviations used are: TTF-1, thyroid transcription factor-1; HD, homeodomaine; Tg, thyroglobulin; βGal, β-galactosidase; CMV, cytomegalovirus; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; TSH, thyroid-stimulating hormone; Luc, luciferase; ADH, alcohol dehydrogenase.

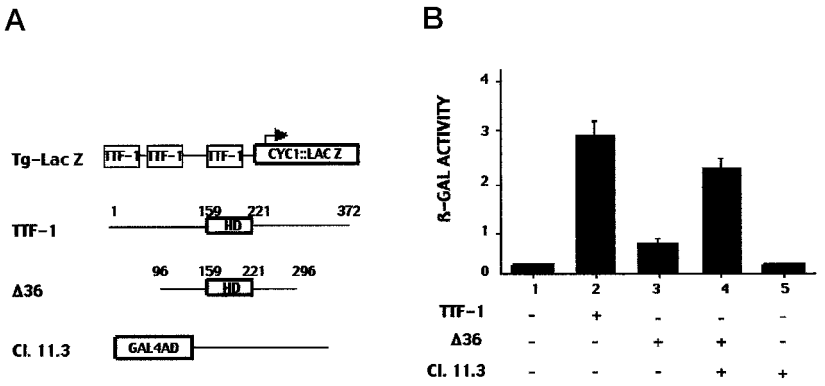


FIG. 1. **Calreticulin interacts with TTF-1 in yeast.** *Panel A*, the structure of the constructs used in the modified yeast one-hybrid system is shown. Tg-lacZ is the thyroglobulin promoter upstream region, in which the TTF-1 binding sites are indicated, fused to the CYC minimal promoter upstream of the *lacZ* cistron; TTF-1 is schematic structure of the TTF-1 protein, with HD indicating the TTF-1 homeodomain; Δ36 is the TTF-1 deletion used for the modified one-hybrid system; cl 11.3 is the clone encoding calreticulin fused to the GAL4 activation domain (*GAL4 AD*) isolated in the modified one-hybrid screen. *Panel B*, INVSC 1 yeast cells were transformed with the reporter plasmid Tg-lacZ. Single colonies were then transformed with expression vectors encoding the indicated proteins. β-Galactosidase activity was measured in triplicate by quantitative liquid culture assay. *Bars* indicate the S.D.

Yeast One-hybrid System—A yeast strain containing both the reporter plasmid Tg-βGal and the ADH1Δ36 bait was constructed in INVSC1 yeast cells and maintained by selection on Ura^-Trp^- medium. These cells were used for transformation with an FRTL-5 cDNA expression library, constructed in the GAL4 activation domain plasmid pGAD10 (CLONTECH). After transformation, cells were plated on $Ura^-Trp^-Leu^-$ agar medium. The screening was performed by β-galactosidase activity using a colony color assay as described (CLONTECH, Matchmaker two-hybrid system protocol).

In Vitro Transcription and Translation—1 μg of CMVcalreticulin was transcribed and translated using the TnT®-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions.

In Vitro Protein Binding—Both full-length TTF-1 as well the NH_2-terminal coding sequence were expressed in *E. coli* as fusion proteins with a 6-histidine tail and purified as described (28, 35). Expression and purification of TTF-1 HD were done as reported (36). For the *in vitro* protein binding, 80 μg of purified full-length TTF-1 was incubated with 0.2 ml of Ni^+-nitrilotriacetic acid-agarose resin (Qiagen) for 30 min at 4 °C in a final volume of 2 ml of buffer B250 (25 mM Tris/Cl, pH 7.5, 15 mM $MgCl_2$, 0.15 mM EGTA, 0.3% Triton X-100, 1 mM dithiothreitol, 250 mM NaCl, and the protease inhibitors leupeptin, pepstatin, and phenylmethylsulfonyl fluoride). After incubation the resin was washed twice with 2 ml of B250 and resuspended in 2 ml of B250. Each *in vitro* translated [^{35}S]methionine-labeled protein was incubated with 0.1 ml of resin containing either TTF-1 protein or BSA. After incubation, the resins were washed using different salt concentration (B250, B500, B700). Elution was carried out with 40 μl of 2 × SDS loading buffer. The eluted proteins were run on a 12% denaturing SDS-polyacrylamide reducing gel. After fixation for 20 min with 10% acetic acid and 10% methanol, the gel was enhanced with an Enlightning solution (NEN Life Science Products), dried, and exposed for autoradiography.

An *in vitro* protein binding assay with deletion mutants of TTF-1 was performed using both the TTF-1 NH_2-terminal domain and TTF-1 HD linked to CNBr-activated Sepharose 4B resin (Amersham Pharmacia Biotech Inc.) at a final concentration of 1 mg of protein/ml of resin. As control, BSA was linked to CNBr-activated Sepharose 4B resin at the same final concentration. 0.2 ml of packed resin containing the immobilized proteins was preincubated with 2 ml of B250 for 30 min at 4 °C. The resin was washed twice and resuspended in 2 ml of B250. 0.1 ml of B250 resin was incubated with *in vitro* translated [^{35}S]methionine-labeled calreticulin. The *in vitro* protein binding assay was carried out as described for full-length TTF-1 protein.

Cell Culture and Transfection—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transient expression assay, cells were plated at 5 × 10^5 cells/60-mm tissue culture dish 4–6 h before transfection. Transfections and measurement of luciferase activity on cell extracts were determined as described (29). CAT expression was detected using a CAT enzyme-linked immunosorbent assay (Boehringer Mannheim) according to the manufacturer's instructions. The thyroid cell line PC was maintained as described in a medium supplemented with thyroid-stimulating hormone (TSH) and insulin (37), except when otherwise stated.

In Vitro Folding and Band Shift Assay—Thermal denaturing of TTF-1 HD was performed in 40 mM Hepes, pH 7.9, and 1 mg/ml BSA for 10 min at 60 °C followed by fast cooling in ice for 3 min. Native and heat-treated TTF-1 HD were incubated with increasing amounts of *in vitro* translated calreticulin for 15 min at room temperature. Incubation with reticulocyte lysate alone was used as control. The DNA binding activities of 2 ng of native and denatured TTF-1 HD, before and after incubation with calreticulin, were measured by band shift assay using oligonucleotide C (38).

Northern Blot Analysis—15 μg of total RNA, prepared from PC cells maintained under different conditions (37), was electrophoresed in 1% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N-plus membrane (Amersham Pharmacia Biotech Inc.). The blot was hybridized with a ^{32}P-labeled *Hin*dIII/*Bam*HI fragment of the plasmid CMVcalreticulin, encoding calreticulin from amino acid 1 to 193. To normalize for RNA loading, hybridization with ^{32}P-labeled TTF-1 (38) and β-actin (39) probes was carried out.

RESULTS

Isolation of cDNA Clones Encoding Proteins That Interact with TTF-1—We used a modification of the yeast one-hybrid system to identify TTF-1-interacting proteins. The indicator yeast strain carries a chimeric gene where transcription of a *lacZ* reporter is under the control of a segment of the thyroglobulin (Tg) promoter fused to the minimal yeast CYC-1 promoter (Fig. 1*A*). The portion of the Tg promoter used contains three TTF-1 binding sites and has been shown to function as an enhancer (31). Furthermore, we have demonstrated previously that this Tg promoter segment can be activated efficiently by TTF-1 in mammalian non-thyroid cells (29). This reporter showed little background activity in yeast, but it was transactivated efficiently by full-length TTF-1 (Fig. 1*B*).

As bait, we expressed in the indicator strain a deletion mutant of TTF-1 (Δ36, amino acids 96–296) which is not able to transactivate the reporter gene (Fig. 1, *A* and *B*). Δ36 is also unable to activate transcription from the Tg promoter in mammalian cells (29) but can compete with the full-length protein. Because Δ3, another TTF-1 deletion that includes the homeodomain, is unable to show the same competing activity (29), we hypothesize that Δ36 does not compete with TTF-1 for the DNA binding site but for a common coactivator.[2] The indicator cells were then transformed with a FRTL-5 cDNA library constructed in a pGAD10 vector, in which each cDNA clone is fused to the NH_2-terminal end of the GAL4 transcriptional activation domain. GAL4AD-cDNA fusion proteins, which can interact with Δ36 or bind directly to the Tg promoter, will

[2] M. De Felice, personal communication.

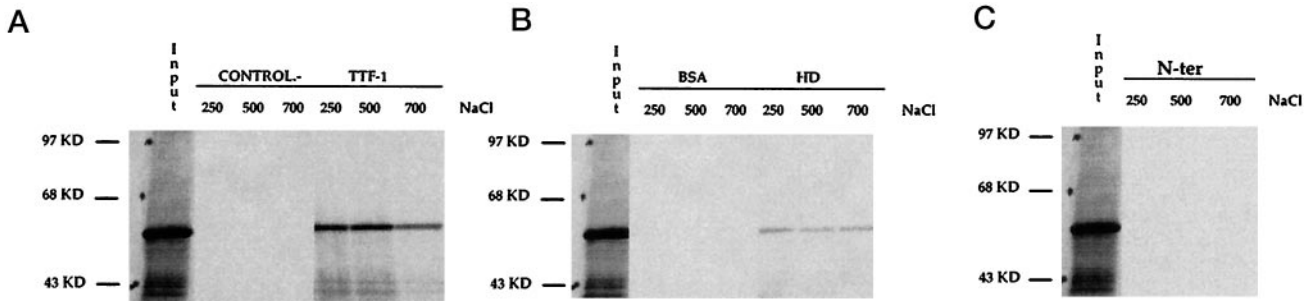


FIG. 2. ***In vitro* binding between calreticulin and TTF-1 or TTF-1 deletion mutants.** *In vitro* translated, [^{35}S]methionine-labeled calreticulin was incubated with immobilized full-length TTF-1 (*panel A*), TTF-1 HD (*panel B*), or the TTF-1 NH_2-terminal domain (*panel C*). After incubation the resins were washed using 250, 500, and 700 mM NaCl. Bound proteins were eluted with SDS loading buffer and resolved by SDS-polyacrylamide gel electrophoresis. Full-length TTF-1 is a 6 histidine-tagged protein, immobilized on a nitrilotriacetic acid resin. Control is the resin without protein. The two TTF-1 deletions (HD) and NH2 terminal domain (*N-ter*) were immobilized on CNBr-activated Sepharose. The same resin coupled to BSA was used as control.

activate transcription of *lacZ* and will show a blue phenotype by colony color assay. A screen of 1×10^6 primary transformants yielded nine potential positive clones. All but three of these could activate the reporter gene upon transformation into yeast lacking Δ36, and they were discarded. In contrast, three plasmids could only activate the reporter gene in the presence of Δ36. Sequence analysis showed that one of the plasmids (clone 11.3) contained a cDNA insert encoding calreticulin.

As shown in Fig. 1*B*, coexpression of GAL4AD-calreticulin fusion protein encoded by clone 11.3, together with Δ36, resulted in increased reporter gene activity. This increased activity was dependent upon the presence of the bait, as it was not observed when the GAL4-calreticulin hybrid was expressed alone.

In Vitro Association of TTF-1 and Calreticulin—To confirm the specific interaction between calreticulin and TTF-1 observed in yeast cells, we first examined the interaction between TTF-1 and calreticulin *in vitro*. Full-length TTF-1 was expressed in *E. coli* as a fusion protein with a 6-hystidine residue tag (35). The fusion protein was immobilized on a Ni^+-nitrilotriacetic acid-agarose resin and incubated with *in vitro* translated, [^{35}S]methionine-labeled calreticulin. To analyze the strength of interaction, we measured the amount of calreticulin bound on TTF-1 after washing with a buffer of increasing ionic strength. As shown in Fig. 2*A*, calreticulin binds to TTF-1 but fails to associate with a control resin, and it is not able to bind the unrelated protein TTF-2 (data not shown). The interaction between calreticulin and TTF-1 seems to be strong, as shown by the resistance of the complex to a 700 mM NaCl wash.

To establish which region of TTF-1 interacts with calreticulin, we immobilized TTF-1 HD (36), the TTF-1 NH_2 terminus (28), and BSA as a negative control on CNBr-activated Sepharose 4B resin. The different resins were incubated with *in vitro* translated, [^{35}S]methionine-labeled calreticulin. Incubation and washing conditions were the same as described above for the binding assay with full-length TTF-1. As shown in Fig. 2*B*, calreticulin is able to bind TTF-1 HD, although it fails to interact with the TTF-1 NH_2 terminus (Fig. 2*C*) and with a control resin containing immobilized BSA (Fig. 2*B*). The binding of calreticulin to TTF-1 HD *in vitro* is consistent with the data obtained in the yeast one-hybrid system because the bait Δ36 used in yeast contains the entire TTF-1 HD and 63 amino acids of the NH_2-terminal domain.

Calreticulin Increases TTF-1 Activity in HeLa Cells—To assess the *in vivo* significance of the TTF-1-calreticulin interaction shown *in vitro* and in yeast, we determined whether overexpression of calreticulin in HeLa cells would modulate TTF-1 transcriptional activity. We transfected HeLa cells with a plasmid containing Tg minimal promoter fused to a CAT coding sequence (pTACAT3) (31). This promoter showed very little activity in HeLa cells, as demonstrated previously (29), but it was transactivated efficiently upon cotransfection with a TTF-1 expression vector (CMVTTF-1) containing the TTF-1 cistron under the control of the human cytomegalovirus promoter (29). As shown in Fig. 3*A* (*lane 4*), cotransfection of a calreticulin expression vector resulted in an approximately 3-fold increase of TTF-1-induced CAT expression. This increased activity was dependent upon the presence of TTF-1 because there was no effect of calreticulin alone on Tg promoter transcription (Fig. 3*A*, *lane 2*). To provide further support for the direct role of calreticulin on TTF-1 activity, we cotransfected in HeLa cells the CAT reporter plasmid under the control of the C5E1b promoter, which contains five TTF-1 binding sites upstream of the E1b TATA box (29). This promoter is transcribed efficiently only upon cotransfection of the TTF-1 expression vector (Fig. 3*B*, *lane 3*), and CAT expression is still increased 3-fold when calreticulin is overexpressed (Fig. 3*B*, *lane 4*).

Because calreticulin binds to the TTF-1 HD, we asked whether this interaction is necessary for the observed calreticulin-mediated increase of TTF-1 activity. To this end we tested the effect of calreticulin overexpression on the activity of TTF-1 deletion mutants fused to the DNA binding domain of GAL4. These TTF-1-GAL4 fusions were tested on the G5E1b reporter plasmid (40) containing five GAL4 binding sites upstream of the E1b TATA box. It has been demonstrated previously that TTF-1 has two distinct activation domains and that either the entire region NH_2-terminal to the homeodomain (mutant ΔG7) or a segment of TTF-1 from the COOH-terminal (mutant ΔG13) fused with the DNA binding domain of GAL4 is able to activate the expression of the G5E1b reporter, containing five DNA binding sites for GAL4 (29). We tested the role of calreticulin overexpression on both ΔG7 and ΔG13 fusions. The experiment revealed that both fusions are insensitive to calreticulin overexpression (Fig. 4*B*), suggesting that the target of calreticulin function is the TTF-1 HD.

Calreticulin Is Able to Refold HD in Vitro—Because the calreticulin-TTF-1 interaction resulted in an increased activity of TTF-1 and calreticulin binds TTF-1 HD *in vitro*, we asked whether calreticulin can exert its effect by modulating the DNA binding activity of TTF-1 HD. It has been demonstrated previously that the helical content of TTF-1 HD is very sensitive to temperature variation and that this thermal denaturing resulted in a greatly decreased DNA binding activity of TTF-1 HD (36). Thus, we tested the capability of calreticulin to improve the refolding of TTF-1 HD after heat denaturing. To this

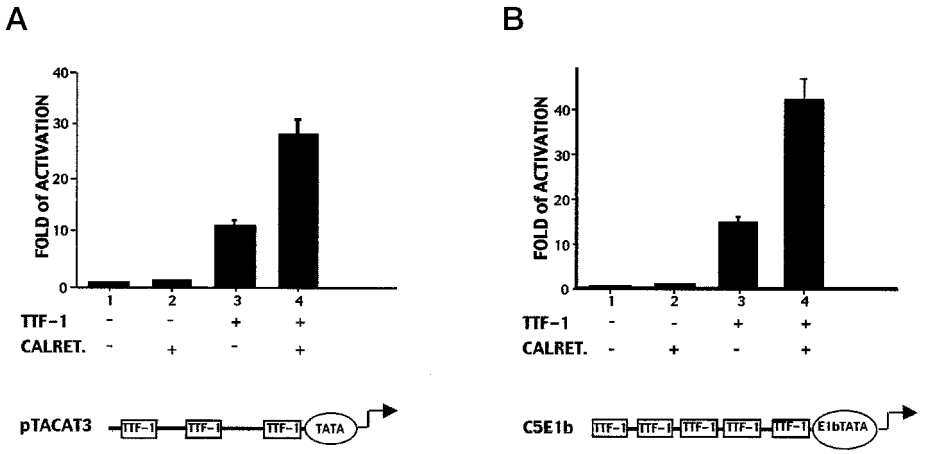
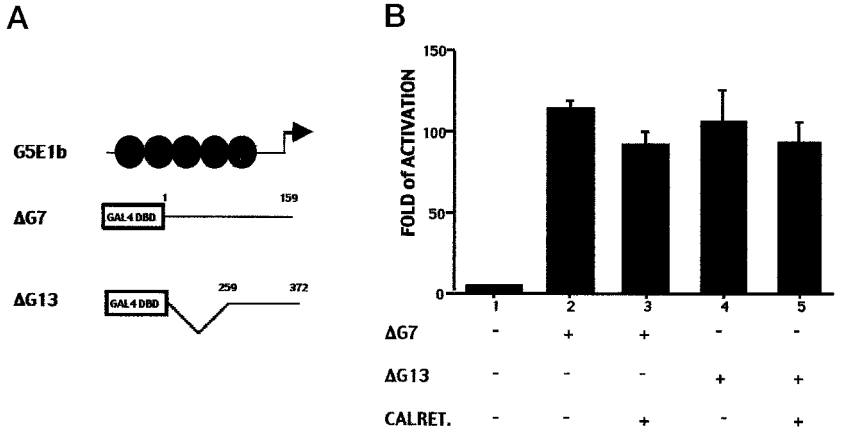


FIG. 3. **Effect of overexpression of calreticulin in HeLa cells on TTF-1 activity.** *Panel A*, HeLa cells were transiently transfected with the reporter plasmid pTACAT3 (3 μg) and expression vectors encoding TTF-1 (0.25 μg) and calreticulin (2.5 μg) as indicated. CMV-Luc was cotransfected to normalize for transfection efficiency. Values are expressed as the fold of activation of the reporter gene above that observed with the reporter alone and represent the average of three independent experiments. The *error bars* show the S.D. for the mean. *Panel B*, HeLa cells were transiently transfected with the reporter plasmid C5E1b (3 μg) and expression vectors encoding TTF-1 (0.25 μg) and calreticulin (2.5 μg) as indicated. Values are presented as in *panel A* and represent the average of three independent experiments. The *error bars* show the S.D. for the mean. The structure of the reporters used is illustrated in the *bottom* part of the figure.

FIG. 4. **Effect of calreticulin overexpression on TTF-1 activation domains.** *Panel A*, structure of the reporter construct and of the activators containing the DNA binding domain of GAL4 fused either to the amino- (ΔG7) or the carboxyl- (ΔG13) activation domains of TTF-1. *Numbers* refers to the amino acid residue in the TTF-1 protein. *Panel B*, HeLa cells were transiently transfected with the reporter plasmid G5E1b (3 mg) and expression vectors encoding ΔG7 (0.1 μg), ΔG13 (0.25 μg), and calreticulin (2.5 μg), as indicated. CMV-Luc was cotransfected to normalized for transfection efficiency. Values are expressed as the fold of activation of the reporter gene above that observed with the reporter alone and represent the average of three independent experiments. The *error bars* show the S.D. of the mean.



end, TTF-1 HD was first heat treated at 60 °C and then incubated at room temperature either with or without an increasing amount of *in vitro* translated calreticulin. The folding state of TTF-1 HD was detected by band shift assay using the oligonucleotide C specifically recognized by TTF-1 (38). As shown in Fig. 5, the refolding of TTF-1 HD increases after the addition of an increasing amount of calreticulin, whereas incubation with the control reticulocyte lysate has no effect on the heat-denatured TTF-1 HD. To define better the role of calreticulin on TTF-1 HD, we analyzed by band shift assay the effect of calreticulin on TTF-1 HD not denatured. Native TTF-1 HD was incubated for 10 min with an increasing amount of *in vitro* translated calreticulin, then its affinity for the labeled oligonucleotide C was detected by band shift assay. Fig. 5 shows that the specific binding of TTF-1 HD on DNA does not change after incubation with calreticulin. These data strongly suggest that calreticulin is able to stimulate refolding of denatured TTF-1 HD but is not able to increase the DNA binding activity of the native TTF-1 HD. Calreticulin alone does not bind to oligonucleotide C (Fig. 5, *lane 16*), and the addition of calreticulin to heat-treated TTF-1 HD produces a band comigrating with the untreated native TTF-1 HD (Fig. 5, *lanes 10–12*), suggesting that calreticulin does not remain in the TTF-1 HD·DNA complex. Together, these results indicate that calreticulin is able to affect the folding state of TTF-1 HD but does not bind stably to either the DNA or protein component of the final complex.

Calreticulin Expression in Thyroid Cells Is Regulated by TSH and Insulin—The DNA binding activity of TTF-1 in thyroid cells has been reported to be under TSH control because of redox regulation (19) even though TTF-1 mRNA appears to be down-regulated by the hormone (41, 42). To investigate whether calreticulin can contribute to hormonal regulation of the DNA binding activity of TTF-1, we measured calreticulin mRNA levels in PC thyroid cells cultured in medium with or without TSH and insulin for different times. As shown in Fig. 6, calreticulin expression is reduced strongly after just 1 day of depletion of hormones, and it is reexpressed 12 h after the addition of TSH and insulin. The variation of calreticulin mRNA levels is specific, as demonstrated by the different effects of the hormonal starvation on β-actin and TTF-1 mRNA. These data suggest that calreticulin could be involved in the hormonal regulation of DNA binding activity of TTF-1.

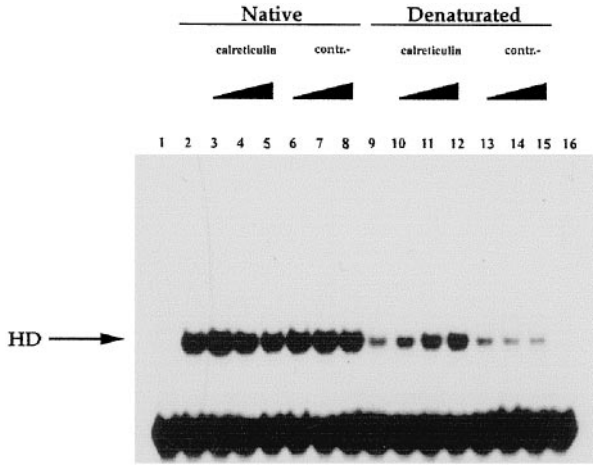


FIG. 5. **Calreticulin refolds TTF-1 HD *in vitro*.** TTF-1 HD, either native (*lanes 2–8*) or heat-denatured (*lanes 9–15*), was incubated with ^{32}P-labeled oligonucleotide C in the presence or absence of *in vitro* translated calreticulin, as indicated. As a control, the same amount of reticulocyte lysate, without calreticulin, was used. *Lane 1* is the free probe. *Lane 16* is calreticulin-containing reticulocyte lysate alone.

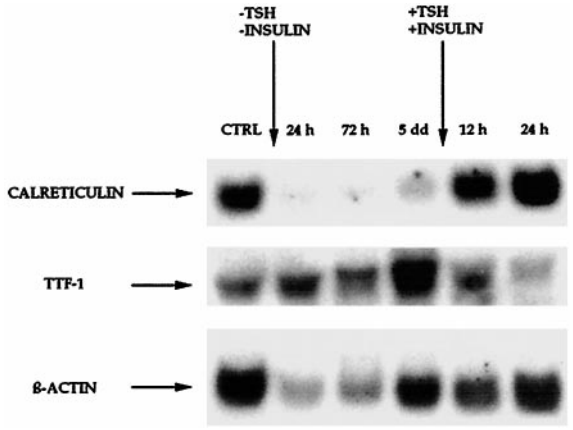


FIG. 6. **Expression of calreticulin mRNA in thyroid cells.** PC cells, grown in the presence of TSH and insulin (*CTRL*), were depleted of both hormones for 24 h, 48 h, 72 h, and 5 days. After 5 days of starvation, the hormones were added again, and cells were cultured for 12 and 24 h. Total RNA was extracted at all time points, electrophoresed on formaldehyde-agarose gel, and transferred to nylon membrane. Hybridization was carried out with a probe encoding the NH_2-terminal-specific domain of calreticulin. To control for RNA, loading hybridizations with probes recognizing β-actin and TTF-1 mRNAs were carried out.

DISCUSSION

We show in the present paper that calreticulin binds to the homeodomain of TTF-1 and is capable of stimulating its refolding. We suggest that the ability of calreticulin to promote renaturing of the TTF-1 HD may be responsible for the observed stimulation by calreticulin of TTF-1 transcriptional activity in cotransfection experiments, perhaps by increasing the steady-state concentration of properly folded homeodomain. The hypothesis that the stimulation of TTF-1 activity by calreticulin depends on its interaction with the homeodomain is supported by experiments showing no effect of calreticulin overexpression on the activity of the two transactivation domains of TTF-1 fused to the heterologous DNA binding domain of GAL4. Calreticulin functions as a Ca^{2+}-binding protein in the endoplasmic reticulum (43), but it has also been found in the nucleus (44), suggesting a role for this protein in regulation of gene expression. In keeping with this hypothesis, calreticulin has been shown to interact with the K*X*FFKR amino acid sequence present in the DNA binding domain of nuclear hormone receptors and to inhibit their DNA binding activity (15, 16). Furthermore, overexpression of calreticulin in cultured cells interferes with transcriptional activation mediated by several nuclear hormone receptors, thus indicating that calreticulin is an important negative coregulator of gene expression (15, 16, 45–48). At variance, however, from the negative interference exerted by calreticulin on transcriptional activation by nuclear hormone receptors, coexpression of TTF-1 and calreticulin results in transcriptional stimulation, both in yeast and in mammalian cells, suggesting that in the case of TTF-1 calreticulin operates with a different mechanism. Furthermore, even though calreticulin interacts also in the case of TTF-1 with its DNA binding domain, the TTF-1 HD lacks the sequence K*X*FFKR, suggesting that in this case calreticulin recognizes a different structural motif. The positive effect of calreticulin on the transcriptional activity of TTF-1 parallels the stimulation of DNA binding which was observed only on the thermally denatured homeodomain. In this respect it could be of relevance the observation that the TTF-1 HD appears to be more sensitive than other homeodomains to thermal denaturing, a property that has been related to the peculiar DNA binding specificity of this homeodomain (36, 49). Thus, it is conceivable that the DNA binding activity of the TTF-1 HD, and perhaps other HDs of the same NK-2 class which show similar properties (50, 51), could be regulated by mechanism that impinges on their peculiar flexibility. The conformational activation of TTF-1 HD seems to result from a transient interaction between calreticulin and unfolded TTF-1 HD. In fact, in an *in vitro* assay, calreticulin is not found associated with the TTF-1 HD·DNA complex. These data are similar to those demonstrating that HSP90 is able to activate the DNA binding potential of MyoD1 by a transient interaction with it (17). However, the *in vivo* assay in yeast cells showing TTF-1-dependent stimulation of transcription by the GAL4AD-calreticulin fusion protein suggests that this interaction could be stable enough to activate transcription *in vivo* but not to survive the conditions of the DNA binding assay. Alternatively, the interaction between calreticulin and TTF-1 could be stabilized *in vivo* by additional factors that are absent in our assay.

Homeodomains have their primary function in the recognition of specific DNA sequences (52). However, this function can be highly regulated, mostly as consequence of protein-protein interactions. For example, the specific DNA sequence that is recognized by a given homeodomain can be changed by the interaction with other homeodomain-containing proteins (53, 54). Furthermore, homeodomains have been shown to interact with non-homeodomain proteins (55, 56), thus expanding the protein-protein interaction functions of this highly regulated protein domain. The interaction with calreticulin which we report in this study could be of a general relevance, and it could be instrumental in regulating the amount of functional homeodomain present in the cell by regulation of its folding. In this respect it is of interest that calreticulin expression in thyroid cells is highly and rapidly regulated by TSH and insulin. A regulation of calreticulin gene expression by the cAMP pathway has already been reported in mouse melanoma cells (46). In thyroid cells TSH and insulin have been demonstrated to stimulate the expression of the thyroglobulin gene (39), whose promoter is activated by TTF-1 (31). Previous studies have shown that TTF-1 is redox-regulated and that the redox state of TTF-1 is TSH-dependent (18, 19). The phosphorylation of TTF-1 also seems to be regulated by hormones (21, 23). The regulation of the folding state of TTF-1 HD by calreticulin could be an additional mechanism involved in hormonal control of gene expression in thyroid cells.

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Calreticulin Enhances the Transcriptional Activity of Thyroid Transcription Factor-1 by Binding to Its Homeodomain

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