TSH controls Ref-1 nuclear translocation in thyroid cells

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ABSTRACT

Ref-1 (called also APE) is a bifunctional protein playing a role in a large variety of cell functions. It is a major member of the DNA base excision repair system. Moreover, through reduction of cysteine residues, Ref-1 controls the activity of several transcription factors. It has been previously demonstrated that TSH up-regulates Ref-1 gene expression in thyroid cells. By using the rat FRTL-5 cell line, we demonstrate that TSH controls Ref-1 intracellular localization. Western blot experiments indicate that addition of TSH to the culture

medium increases the Ref-1 cytoplasm-to-nucleus translocation. This phenomenon occurs at early times of TSH stimulation and is not dependent on protein neosynthesis. The Ref-1 cellular compartmentalization was also investigated in human thyroid tumors. A Ref-1 nuclear/cytoplasmic ratio difference between normal and cancerous thyroid tissues was observed. These results suggest that Ref-1 localization may have a critical role in the control of thyroid cell functions.

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INTRODUCTION

Ref-1, also called APE, is a protein having both apurinic/aprimidinic endonuclease DNA repair activity and nuclear redox activity (Nakamura *et al.* 1997, Rothwell *et al.* 1997). This protein plays a fundamental role in DNA base excision repair and its activity is considered as the rate-limiting step in DNA repair due to oxidative damage (Ramana *et al.* 1998). By means of the redox activity, Ref-1 is able to control the DNA-binding function of several transcription factors, including AP-1, NF- κ B, ATF/CREB and Pax proteins. (Xanthoudakis *et al.* 1992, Tell *et al.* 1998a). Recent developments have also intimately linked Ref-1 as a major controlling factor for p53 activity through a redox mechanism (Jayaraman *et al.* 1997) and have established its functional interaction with p53 *in vivo* (Meira *et al.* 1997, Gaiddon *et al.* 1999). There are several pieces of evidence demonstrating the biological relevance of Ref-1. The down-regulation of Ref-1 protein levels, through antisense techniques, increases DNA damage in cells treated with various agents

(Walker *et al.* 1994). The Ref-1 gene inactivation in mice is lethal at early stages of embryonic development (Xanthoudakis *et al.* 1996). Both DNA repair mechanisms and some of the transcription factors regulated by Ref-1 are heavily involved in the generation/progression of human cancer. Thus, it could be conceivable that Ref-1 may have a role during neoplastic transformation.

Since Ref-1 controls the binding activity of a large variety of transcription factors, it could represent an important device for regulation of gene expression. Accordingly, Ref-1 protein is up-regulated by non-toxic levels of a variety of reactive oxygen species (ROS) such as superoxide anion (O₂⁻), H₂O₂ and the hydroxyl radical (OH[•]), which can be generated by external agents such as ionizing radiation (Ward 1994). Moreover, ROS can be produced during pathological states in activated neutrophils and by treating cells with cytokines such as tumor necrosis factor- α and interleukin-1 β (Schreck *et al.* 1991). Ref-1 up-regulation induced by ROS is due to translational mechanisms, being blocked by the treatment of cells with

cycloheximide (Ramana *et al.* 1998). Ref-1 is subjected to hormonal control; in thyroid cells Ref-1 protein is up regulated by thyrotropin (TSH) (Asai *et al.* 1997). The regulation of transcription factors by Ref-1 occurs through a post-translational mechanism (reduction of cysteine residues) that operates upon preformed proteins and that, therefore, is much faster than mechanisms acting through protein neosynthesis. From a regulatory point of view, these data generate an apparent paradox. In fact external stimuli, such as cytokines or TSH, would activate a fast mechanism of gene expression regulation (reduction of cysteine residues in DNA-binding domains of transcription factors) by a time-consuming event such as the neosynthesis of the regulator (Ref-1).

Based on these considerations, the focus of our investigation was to test whether TSH controls Ref-1 activity by mechanisms different from protein neosynthesis. We demonstrate that TSH increases the Ref-1 cytoplasm-to-nucleus translocation suggesting that the cell localization of this protein may define different functional states of the thyroid cell. For these reasons the Ref-1 cellular localization in human thyroid tumors was investigated. A Ref-1 nuclear/cytoplasmic ratio difference between normal and cancerous thyroid tissues was observed.

MATERIALS AND METHODS

Rat FRTL-5 cell culture and cell fractionation

FRTL-5 cells were cultured as described (Ambesi-Imbiombato & Coon 1979). Cell nuclear extracts were prepared as previously described (Tell *et al.* 1998a). Briefly, 10^7 cells were washed once with PBS and resuspended in 500 µl hypotonic lysis buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM MgCl₂, 0.1 mM EDTA, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 0.5 mM polymethylsulfonyl fluoride (PMSF), pH 7.9). After 10 min, cells were homogenized by ten strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation for 5 min at 500 g at 4 °C in a microcentrifuge. The supernatant obtained after this centrifugation was considered as the cytoplasmic fraction. Nuclear proteins were extracted with 100 µl buffer B (10 mM Hepes, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 0.5 mM PMSF, pH 7.9). After incubating for 20 min at 4 °C, samples were centrifuged at 12 000 g at 4 °C for 15 min. Nuclear and cytoplasmic extracts were then quantitated for protein levels (Bradford 1976) and used immediately for Western blot analysis or kept at –80 °C.

Western blot analysis

Twenty micrograms nuclear or cytoplasmic extracts, obtained from FRTL-5 cells incubated under different conditions, were electrophoresed in a 10% SDS-PAGE. Then proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). After transfer, membranes were saturated by incubation, at 4 °C overnight, with 10% non-fat dry milk in PBS/0.1% Tween-20 and then incubated with the rabbit anti-Ref-1 antibody for 60 min at room temperature (RT). The Ref-1 affinity-purified polyclonal antibody was used as previously described (Xu *et al.* 1997). After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled to peroxidase (Sigma Chemical Co., St Louis, MO, USA). After 60 min incubation at RT the membranes were washed several times with PBS/0.1% Tween-20 and the blots were developed using an enhanced chemiluminescence procedure (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Tissue samples

Twenty-three formalin-fixed, paraffin-embedded thyroid tissue samples were evaluated for the expression of Ref-1 protein using an immunoperoxidase technique.

Tissue samples included four cases of follicular adenomas, five of papillary carcinoma, five of follicular carcinoma and four of undifferentiated carcinoma. The surrounding unaffected tissues from five cases of follicular adenomas were obtained as representative specimens of normal thyroid. Representative blocks of each case were selected for immunohistochemical staining.

Immunohistochemistry

Immunohistochemical detection of Ref-1 protein was performed by the avidin-biotin-peroxidase (ABC) method (Vectastain ABC Elite Kit; Vector, Burlingame, CA, USA), using a polyclonal anti-Ref-1 antibody produced at a 1:50 dilution.

The sections were freshly cut immediately before immunostaining, and placed on glass slides coated with 3-aminopropyltriethoxysilane. After deparaffinization by sequential passages through xylene, graded ethanol, and deionized water, the sections were boiled in 0.1 M citrate buffer, pH 7.3, in a 750 W microwave oven set at maximum power for 2 min and at 210 W for 8 min. The sections were incubated with the above antibodies for 60 min at RT, then incubated with a secondary biotinylated antibody for 30 min at RT, followed by ABC

reagent for 30 min at RT. The reaction product was detected with 3,3'-diaminobenzidine (Sigma). The sections were counterstained with hematoxylin. For negative controls, the primary antibody was omitted and replaced with rabbit immunoglobulins (Dako, Denmark). Cells were considered Ref-1-positive when brown staining of the nucleus and/or the cytoplasm was identified. One thousand cells for each case were evaluated by determining the nuclear/cytoplasmic positivity.

RESULTS

Hormonal control of Ref-1 cytoplasm-to-nucleus translocation

In normal thyroid cells Ref-1 is localized in both the cytoplasm and nuclear compartments (Kakolyris *et al.* 1998). We decided to test, therefore, if the Ref-1 cytoplasm-to-nucleus translocation is controlled by TSH in thyroid cells. The thyroid FRTL-5 cell line was used (Ambesi-Imbiombato & Coon 1979). The relative amount of Ref-1 protein was assayed by Western blot analysis after the separation of nuclear and cytoplasm compartments. Fig. 1A shows the Ref-1 signal detected in cytoplasm or nucleus in cells cultured: (i) in the presence of TSH (1 mU/ml); (ii) in the absence of TSH for 5 days; and (iii) in cells cultured in the absence of TSH for 5 days and then hormonally stimulated for 1, 3 and 6 h. Signals from several experiments have been quantitated by scanning densitometry and results are shown in Fig. 1B. Cells cultured in the presence of TSH showed a strong Ref-1 signal in both the nuclear and cytoplasmic fraction. In the absence of TSH, Ref-1 levels decreased in both fractions but the reduction was much more evident in the cytoplasmic fraction. Compared with unstimulated cells, after 1 h of TSH treatment, an increase in the nuclear Ref-1 was detected, and it was associated with a reduction in the cytoplasmic fraction. After 3 or 6 h of TSH treatment the amount of nuclear Ref-1 was similar to that detected after 1 h of TSH stimulation. On the contrary, Ref-1 cytoplasmic levels at these late times were much more increased compared with those observed after 1 h. These results suggest that the most significant effect, at early times of TSH stimulation, is an accelerated rate of Ref-1 cytoplasm-to-nucleus translocation. Only at late times (3 and 6 h) is the TSH effect on Ref-1 neosynthesis significant. Due to this two-stage mode of stimulation by TSH, one would predict that an increase of nuclear Ref-1 levels upon TSH addition should be observed in conditions of blocked protein synthesis. In order to verify this

FIGURE 1. Hormonal control of Ref-1 cytoplasm-to-nucleus translocation. (A) FRTL-5 cells were cultured in the absence of TSH (5H) for 5 days (lanes 1 and 2), in the presence of 1 mU/ml of TSH for different times ranging from 1 to 6 h (lanes 3–8), and constitutively treated with TSH (6H) without starvation (lanes 9, 10). The cells were then processed to obtain cytoplasmic ('C', odd lanes) and nuclear ('N', even lanes) extracts. Twenty micrograms of each extract were loaded onto an SDS-PAGE for Western blot analysis with anti-Ref-1 polyclonal antibody and detected by ECL. (B) Values obtained from densitometric scanning of bands from several experiments are shown as the ratio between the Ref-1 protein levels measured in cells treated with TSH (stimulated) and in untreated cells (control). Open columns represent the cytoplasmic Ref-1 protein levels and hatched columns the nuclear fractions. Bars indicate the mean value $\pm$ s.d. of at least three independent experiments.

prediction, time-course experiments were run in the presence of cycloheximide, resembling conditions of blocked protein synthesis. As shown in Fig. 2, TSH

FIGURE 2. Cycloheximide pre-treatment of FRTL-5 cells does not prevent Ref-1 translocation into the nucleus after TSH treatment. (A) FRTL-5 cells were preincubated in the presence of the protein synthesis inhibitor cycloheximide (10 µg/ml) for 1 h before exposure to TSH. The cells were then processed to obtain cytoplasmic ('C', odd lanes) and nuclear ('N', even lanes) extracts. Twenty micrograms of each extract were loaded onto an SDS-PAGE for Western blot analysis with anti-Ref-1 polyclonal antibody. 5H indicates FRTL-5 cells cultured in the absence of TSH for 5 days. (B) Values obtained from densitometric scanning of bands from experiments of panel (A) are reported as the ratio between the Ref-1 protein levels measured in cells treated with TSH (stimulated) and in untreated cells (control). Open columns represent the cytoplasmic Ref-1 protein levels and hatched columns the nuclear fractions. Bars indicate the mean value±s.d. of at least three independent experiments.

stimulation was still able to increase nuclear Ref-1 levels. Cytoplasmic Ref-1 levels were decreased, as

expected since TSH stimulates Ref-1 cytoplasm-to-nucleus translocation but it was not able to increase Ref-1 protein synthesis (due to the presence of cycloheximide). Therefore, we were able to dissociate the TSH effect due to cytoplasm-to-nucleus translocation from that due to novel protein synthesis.

Ref-1 immunostaining of human thyroid tissues

The observation that the Ref-1 cellular localization is regulated by TSH suggests that the difference in compartmentalization of this protein may have a biological relevance. In order to corroborate this statement, the cellular localization of Ref-1 in human thyroid tumors was investigated. The Ref-1 immunodetection in normal thyroid tissue and thyroid adenomas, as well as in papillary, follicular and undifferentiated thyroid carcinomas is shown in Fig. 3. In normal thyroid tissue (five cases) follicular cells showed a strong nuclear staining. Ref-1 was also detectable in cytoplasm as dot-like structures; the intensity of the signal was much weaker than that detected in the nucleus. Thyroid adenomas showed a nuclear staining superimposable on that observed in normal tissue. However, no cytoplasmic signal was detected in three out of four cases investigated. In papillary (five cases), follicular (five cases) and undifferentiated thyroid carcinomas (four cases) the nuclear Ref-1 signal was less intense than that observed in normal thyroid. No difference could be detected in the cytoplasmic signal among normal tissue, follicular and undifferentiated carcinomas. In papillary carcinomas the cytoplasmic signal was particularly intense and homogeneously diffused. In order to quantitate the Ref-1 distribution in nuclear and cytoplasmic compartments, the ratio between Ref-1 nuclear-positive cells and Ref-1 cytoplasmic-positive cells was determined. Results are shown in Fig. 4. The nucleus/cytoplasm ratio was significantly lower in all histotypes of carcinomas compared with the normal tissue. No difference was detected among the different histotypes of cancer. Altogether, these results indicate that modification of the nuclear/cytoplasmic distribution of Ref-1 occurs in thyroid carcinomas.

DISCUSSION

Recent studies have demonstrated that redox potential-based mechanisms play a critical role in the regulation of transcription factors (Roy *et al.* 1997). Redox control of transcription factors is a

FIGURE 3. Ref-1 localization in normal and pathological human thyroid tissues. Immunohistochemical detection of Ref-1 was performed as described in Materials and Methods. Representative cases are presented (50 × enlargement). The brown staining indicates the presence of Ref-1 protein. (A) Normal thyroid; (B) follicular adenoma; (C) papillary carcinoma; (D) follicular carcinoma; (E) undifferentiated carcinoma.

fast and convenient way to regulate gene expression and provides the cell with the possibility of quickly reacting to external stimuli (Zheng *et al.* 1998). Redox regulation of transcription factors is largely based on the action of Ref-1 protein. Activation of transcription factors by this protein occurs through reduction of cysteine residues in the DNA-binding domain (Sen & Packer 1996). Asai *et al.* (1997) have demonstrated that TSH increases Ref-1 protein levels in thyroid cells. In this study, we have solved

an apparent paradox. In fact, according to published data, the intracellular regulator, Ref-1, activates transcription factors through a fast mechanism (reduction of cysteine residues); however, the external signal, TSH, would activate the regulator through a time-consuming mechanism, neosynthesis of Ref-1. We have demonstrated that the first significant event of Ref-1 activation by TSH is the increase of cytoplasm-to-nucleus translocation, a fast mechanism in keeping with the high-speed

FIGURE 4. Nucleus/cytoplasm positivity ratio in normal and pathological human thyroid tissues. Bars indicate the mean values and standard deviation of the ratios between nuclear-positive and cytoplasmic-positive cells in normal thyroid tissues and thyroid carcinomas. 'Total' indicates the value of the whole set of carcinomas. The significance between normal tissues and carcinomas was determined by a chi-square test.

mechanism by which Ref-1 activates transcription factors (reduction of cysteine residues). In this manner the redox signaling could rapidly modify thyroid gene expression.

In terms of transcriptional regulation, the present results should be coupled with those of our previous work (Tell *et al.* 1998b) where we demonstrated that Ref-1 overexpression is able to enhance the transcriptional activity of the thyroid-specific transcription factor, Pax8. In addition, it has been previously demonstrated that TSH induces prompt Pax8 activation through a rapid increase in its DNA-binding activity (Kambe *et al.* 1996). These authors suggested that the observed regulatory event would be due to thioredoxin, a cytoplasmic reducing enzyme. Here, we suggest that Ref-1 also could be involved in this rapid activation of Pax8.

Several data suggest that the cytoplasm-to-nucleus translocation induced by TSH could have a role in turning on the neosynthesis of Ref-1 protein. In fact, it has been demonstrated that Ref-1 is able to induce c-jun DNA-binding activity (Xanthoudakis *et al.* 1992). Moreover, c-jun is a part of the multiprotein complex that activates Ref-1 promoter (Grösh & Kaina 1999). Thus, one can envisage a simple model in which: (i) TSH enhances Ref-1 cytoplasm-to-nucleus translocation;

(ii) nuclear Ref-1 induces c-jun DNA-binding activity; and (iii) a c-jun-dependent protein-DNA complex activates Ref-1 promoter activity up-regulating Ref-1 protein synthesis. Accordingly, the total amount of Ref-1 protein present in FRTL-5 cells after long-term TSH treatment is much higher than that observed after short (1 h) hormonal stimulation (Tell *et al.* 1999b, and data not shown). By this mechanism, Ref-1-dependent transcriptional regulators can be turned on in a rapid fashion and then maintained on for an extended period of time.

In agreement with a previous investigation (Kakolyris *et al.* 1998), Ref-1 has been detected in both cytoplasm and nucleus in normal thyroid cells. Thyroid tumors do not show elevation of Ref-1 expression compared with normal tissue. On the contrary, increase of Ref-1 expression has been observed in cervical and germ cell tumors (Xu *et al.* 1997, Kelley *et al.* 1998). Compared with normal tissue, thyroid carcinomas show a reduced Ref-1 nucleus/cytoplasm immunoreactivity ratio. These results are reminiscent of those observed in epithelial ovarian cancer (Moore *et al.* 1999). It should be pointed out, however, that in normal ovaries only nuclear staining was observed in the absence of cytoplasm staining. Both nuclear and cytoplasmic staining was detected in ovarian tumors, but in the more aggressive ovarian cancers, the amount of cytoplasmic levels of APE/Ref-1 were dramatically increased and in 50% of cases the nucleus was 'cleared' of Ref-1. Therefore, while in ovarian tumors a qualitative change in Ref-1 localization occurs, in thyroid tumors only quantitative changes have been detected. The lack of difference between papillary, follicular and undifferentiated thyroid carcinomas would suggest that the modification of Ref-1 localization is not related to aggressiveness in thyroid tumors. At present, the functional significance of the altered Ref-1 distribution observed in thyroid carcinomas with respect to normal tissue is not clear. Because of the dual nature of Ref-1 (as regards its redox and repair activity) it would be interesting to investigate the two activities in the different cellular compartments to test whether the altered distribution observed in thyroid tumors may have a functional relevance. Unfortunately, processing of cell fractions from tissues would affect the Ref-1 activities.

Thyroid carcinogenesis is a multistage process with a sequence of specific gene mutations which stepwise increase the aggressiveness of the neoplasm (Williams 1995, Santoro *et al.* 1995, Wynford-Thomas 1997). For example, p53 gene mutation is present almost exclusively in poorly differentiated thyroid carcinomas (Ito *et al.* 1992,

Fagin *et al.* 1993). In this light, the decrease of nuclear Ref-1 observed in differentiated carcinomas could reduce the DNA repair activity of the cell, facilitating the action of genotoxic agents, and thus progression toward a more aggressive phenotype. This hypothesis is supported by several observations indicating a direct relationship between the amount of nuclear Ref-1 and cell resistance to genotoxic agents (Walker *et al.* 1994, Tomicic *et al.* 1997, Grösh *et al.* 1998, Ludwig *et al.* 1998). To investigate the possibility that nuclear Ref-1 levels may protect cells from genotoxic agents we are selecting thyroid tumor cell lines differing from each other in the Ref-1 nuclear levels (G Tell & G Dahante, unpublished observations).

In conclusion, we have demonstrated that a modification of Ref-1 cellular compartmentalization occurs in thyroid carcinomas and that TSH controls the Ref-1 cytoplasm-to-nucleus translocation. In a previous investigation we have demonstrated that Ref-1 up-regulates the Pax8 transcriptional activity (Tell *et al.* 1998b). Together, this evidence suggests that Ref-1 localization may have a critical role in the control of thyroid cell functions.

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