Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 833 (2006) 41–50
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Differential proteomic analysis of nuclear extracts from thyroid cell lines☆

Anna Maria Salzano[a], Igor Paron[c], Alex Pines[c], Angela Bachi[b], Fabio Talamo[a], Nicoletta Bivi[c], Carlo Vascotto[c], Giuseppe Damante[c], Franco Quadrifoglio[c], Andrea Scaloni[a], Gianluca Tell[c,*]

[a] *Proteomics and Mass Spectrometry Laboratory, I.A.B.B.A.M., National Research Council, via Argine 1085, 80147 Napoli, Italy*
[b] *Mass Spectrometry Unit, DIBIT San Raffaele Scientific Institute, Milan, Italy*
[c] *Dipartimento di Scienze e Tecnologie Biomediche, P.le Kolbe 4, Università degli Studi di Udine, 33100 Udine, Italy*

Received 25 July 2005; accepted 18 December 2005
Available online 23 January 2006

Abstract

Nuclear proteins play a major role in controlling cell functions. Differential proteomic analysis of nuclear proteins by combined 2D gel electrophoresis (2D-E) and mass spectrometry procedures can provide useful information to understand the control of cell proliferation and differentiation. To identify proteins involved in dedifferentiation, we used a differential proteomics approach by comparing nuclear extracts from the differentiated rat thyroid cell line FRTL-5 and the derived undifferentiated Ki-mol cell line, obtained by transformation with the Ki-ras oncogene. Thirteen proteins were identified as differently expressed in the nuclear compartment between the two cell lines. RT-PCR analysis performed on seven differently expressed genes showed that only in two cases the difference may be ascribable to a transcriptional mechanism. Since one of the identified proteins, namely apurinic apyrimidinic endonuclease/redox effector factor-1 (APE1/Ref-1), is suspected to play a role in thyroid tumorigenesis, we used a glutathione S-transferase (GST)-pulldown assay coupled to a 2D electrophoretic/matrix assisted laser desorption ionization-time of flight (MALDI-TOF)–mass spectrometry (MS) analysis to detect and identify its interacting partners. We show here that β-actin directly interacted with APE1/Ref-1, as confirmed by co-immunoprecipitation assays and that this interaction was enhanced by oxidative stress on FRTL-5 cells.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Differential proteomics; Thyroid; Differentiation; APE1/Ref-1; Transcriptional regulation; Nucleus

1. Introduction

Cancer is a multi-faceted disease that presents many challenges to clinicians and researchers searching for more-effective ways to combat its devastating effects. One of the main challenges in this investigation is the identification of markers to improve diagnosis, classification and prognosis of tumors as well as to define targets for more-effective therapeutic measures. At present, tumor classification is a complex process based primarily on macroscopic and histological features, though some cancer-related genes have been mainly identified by mutational analysis. However, genetic markers identified so far are of help only in a small fraction of human neoplasms. Tumors with a similar histological appearance can follow significantly different clinical outcomes and show different responses to therapy. Given the wide different biological features of tumors, additional

Abbreviations: Acy1, aminoacylase 1; APE1/Ref-1, apurinic apyrimidinic endonuclease/redox effector factor-1; 2D-E, bidimensional gel electrophoresis; EF-1α, elongation factor-1α; FUSE B, far upstream element binding protein; Gal-1, galectin 1; Gal-3, galectin 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBLP, guanine nucleotide-binding protein β subunit 2-like 1; GST, glutathione *S*-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MS, mass spectrometry; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCP-1 δ, T-complex protein 1, δ subunit; TFC, thyroid follicular cells; Tg, thyroglobulin; TPO, thyroperoxidase; TSH, thyrothropin; TTF-1, thyroid transcription factor 1

☆ This paper was presented at the 2nd IPSo Congress on Proteomics and Genomics, Viterbo, Italy, 29 May to 1 June 2005.

* Corresponding author. Tel.: +39 0432 494311; fax: +39 0432 494301.
E-mail address: gtell@mail.dstb.uniud.it (G. Tell).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.12.025

methods of classification are required. With the completion of the draft sequence of the human genome, there is a great deal of interest in the use of functional genomics, especially gene expression profiling techniques such as DNA microarrays and proteomics, to identify cancer-associated genes and their protein products [1]. Proteomics approaches to tumor marker identification hold the promise of identifying specific proteins or protein modifications in tumor tissues to assist also in individualizing treatment for certain cancers [2,3]. One approach to simplify such a complex purpose is to use in vitro cellular model of tumorigenesis and to focus the attention on particular subcellular districts, which are primarily involved in the control of the cellular differentiation processes.

Most of cell differentiation events are taken at the transcriptional level in the nuclear compartment by action of the so-called tissue-specific transcription factors that specifically control eukaryotic gene expression. These proteins, together with many nuclear ubiquitous factors, contribute to generate the specific pattern of gene expression peculiar of each cell type. Qualitative or quantitative modifications, at this level of cellular control, lead to altered gene expression often resulting in cellular death or transformation [4,5]. Tissue-specific transcription factors are subjected to regulation by different molecular mechanisms, including posttranslational modifications (phosphorylation, acetylation, etc.) as well as interactions with cofactors (coactivators, corepressors) [6,7]. Therefore, to understand regulation of gene expression in a given cell type, it is critical not only to know which transcription factors are expressed in that cell type, but also the functional interactions established between these molecules and other nuclear proteins. The thyroid follicular cell (TFC) is an excellent model to address these issues. In fact: (i) several cell-specific transcription factors have been identified, including thyroid transcription factor 1 (TTF-1), thyroid transcription factor 2 (TTF-2), Pax-8 and Hex, which undergo complex regulatory control mechanisms during the differentiation process and are impaired during neoplastic transformation [8,9] and (ii) transformed cell lines, with different degrees of dedifferentiation, have been derived from normal TFC cell lines [10] and are currently available. Therefore, comparisons between normal and transformed cell lines may give also hints to better understand generation and progression of human tumours. Several regulatory mechanisms, involving regulatory circuits between nuclear ubiquitous proteins and TFC-specific transcription factors have been already identified. For example, both TTF-1 and Pax-8 are subjected to redox regulation by apurinic apyrimidinic endonuclease/redox effector factor-1 (APE1/Ref-1) [9] and to physical and functional interaction with other ubiquitous proteins [11,12]. Although this kind of knowledge is rapidly growing, we are still quite far from delineating functional nuclear networks that control TFC differentiation.

Global protein analysis by proteomics techniques, not requiring a priori knowledge of the proteins involved and describing, at the same time, the multitude of its molecular components, may provide a more complete picture of the biological system under investigation. On this basis, new hypotheses can be formulated and new scientific scenarios can be opened. To identify proteins involved in the control of TFC gene expression, we used a proteomic approach restricted to proteins present in nuclear enriched samples of cellular extracts. A combined 2D gel electrophoresis (2D-E)/mass spectrometry (MS) methodology was applied to identify differences in the expression levels of proteins between the FRTL-5 cell line, which maintains most of the differentiating features and properties of in vivo thyroid cells [13], and the Ki-mol cell line, which is derived from the first one by Ki-ras oncogene transformation and shows a highly undifferentiated and aggressive phenotype [10]. By using this approach, initially focusing on the definition of the only qualitative ('on-off') differences occurring between nuclear enriched proteome of these two cell lines, we identified galectin 1 (Gal-1) and Galectin 3 (Gal-3) as specifically expressed only in the transformed cell line [14]. Now, we completed this work by considering all quantitative differences (cut off: greater than three-fold in expression level) that we were able to detect. Here we show that, besides the previously identified Gal-1 and Gal-3 [14], other seven proteins resulted specifically over-expressed in the nuclear enriched fraction of Ki-mol cells while four proteins were significantly up-regulated in the nuclear fraction of FRTL-5 cells. Moreover, since one of these latter, namely APE1/Ref-1, seems to play a role in thyroid tumorigenesis, we used a GST-pulldown assay coupled to 2D-E and MS analysis to identify its interacting partners. These data open new perspectives in the comprehension of mechanisms of TFC differentiation and of thyroid tumorigenesis.

2. Experimental procedures

2.1. Cell cultures and materials

FRTL-5 cells were maintained in F12 Coon's modified medium with 5% calf serum and hormone mixture, as previously described [13]. Ki-mol cells were grown under the same conditions as the wild-type FRTL-5 cells. The cells were seeded in 100 mm dishes and grown at 37 °C in a humidified atmosphere of 5% (v/v) CO_2. H_2O_2 treatment was performed on cells after 1 h of serum starvation and in medium w/o serum. All the chemicals described below were from Sigma–Aldrich Co. (Milan, Italy) unless otherwise specified.

2.2. Nuclear extracts preparation

Cell nuclear extracts were prepared as previously described [15]. Briefly, 5×10^7 cells were washed twice with 10 ml of PBS and once with MB buffer (400 mM sucrose, 50 mM Tris, 1 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM potassium hydrogen phosphate, pH 7.6 and 0.2% (w/v) BSA) and centrifuged at 1100 rpm/min, at 4 °C, in a Universal 32 R Ettich centrifuge (Ettich Instruments). The pellet was resuspended in MB buffer (400 μl/10^7 cells) and incubated on ice for 20 min. Subsequently, the cells were homogenized by ten strokes with a loose-fitting Dounce homogenizer and centrifuged at 3500 rpm/min for 1 min at 4 °C. The supernatant contained the mitochondria/cytosol/membranes and the pellet enclosed the nuclei. The pellet with the nuclei was suspended in 5 ml of PBS

and centrifuged for 2 min at 3500 rpm/min at 4 °C. The pellet was suspended in 100 μl/10^7 cells of NB buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF) and incubated for 1 h on ice, subsequently homogenized and applied to 5 ml of 30% (w/v) sucrose in NB buffer. After centrifugation at 2000 rpm/min for 10 min at 4 °C, the pellet was washed twice with 3 ml of NB buffer, centrifuged as above, suspended in 500 μl of NB buffer and centrifuged again at 10,000 rpm/min, at 4 °C. Nuclear proteins were extracted with 500 μl of buffer B (10 mM Hepes, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF, pH 7.9). After incubating for 30 min at 4 °C, samples were centrifuged at 12,000 × g for 20 min, at 4 °C. Nuclear extracts were then analyzed for protein content [16] and stored at −80 °C in aliquots. The quality of nuclear fractions was evaluated by testing, through Western blot analysis, for the presence of the nuclear thyroid specific transcription factor Pax-8 and for the presence of the TATA binding protein (TBP).

2.3. Western-blot analysis and antibodies

Thirty micrograms of nuclear and cytoplasmic proteins obtained from different cell lines, were electrophoresed onto a 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). After transfer, membranes were saturated by incubation with 5% (w/v) non-fat dry milk in PBS/0.1% (w/v) Tween-20 (at 4 °C, overnight) and then incubated with the specific primary antibody (diluted 1:1000 in 5% (w/v) non-fat dry milk in PBS/0.1% (w/v) Tween-20) for 60 min, at room temperature. After three washes with PBS/0.1% (w/v) Tween-20, membranes were incubated with the proper secondary immunoglobulin coupled to peroxidase (Sigma, St Louis, MO, USA). After 60 min of incubation, the membranes were washed several times with PBS/0.1% (w/v) Tween-20 and blots were developed using a chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy).

The anti Pax-8 rabbit polyclonal antibody was (αPax8-187) provided by R. Di Lauro (University of Naples, Naples, Italy) for providing the αPax8-187 rabbit polyclonal antibody. The anti-Gal-3 monoclonal antibody A3A12 was provided by Dr. F.T Liu (University of California-Davis Sacramento, CA, USA). The anti EF-1α monoclonal antibody (clone CBP-KK1) was from Upstate Biotech. (Lake Placid, NY, USA). The anti β-actin monoclonal antibody was from Sigma–Aldrich Co. (St Louis, MO, USA). The polyclonal and monoclonal anti-APE1/Ref-1 antibodies were a gift from Dr. M.R. Kelley (University of Indiana, Indianapolis, IN, USA) and were used as described previously [17,18]. The anti-TBP and anti Gal-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. RT-PCR analysis

Total RNA was purified from FRTL-5 or Ki-mol rat thyroid cells by using the SV Total RNA Isolation System from Promega (Promega, WI, USA) according to the manufacturer's protocol. RT-PCR was performed with 1 μg of total RNA as template. PCR reactions with the specific primers pairs designed to amplify partial cDNAs for some of the identified genes (Table 1) were performed in semi-quantitative fashion to allow comparison of

Table 1
Primers used for RT-PCR analysis of some of the proteins identified by 2D-E

Protein	Accession number	Primer name	Amplification product (bp)	Primer sequence
RAN	P62827	RAN Fw	339	5′-CAGGGAGAGCCGCAGGTCCAGTTCAA-3′
		RAN Rev		5′-TTCACACACGCGTACCAGATCTCTATGCCAG-3′
EF-1α	Q64718	EF1A Fw	689	5′-AGTTTGAGAAGGAGGCTGCGGAGATGGG-3′
		EF1A Rev		5′-ACCAGTTTCCACTCGGCCCACAGGG-3′
FUSE BP	Q12828	FUSE Fw	632	5′-AGCAGCAGCTATGGGCAACCCCAGAGT-3′
		FUSE Rev		5′-GCCTCACCCTTCAACTTGCCAGTTTCCC-3′
Ras	P46203	Ras Fw	441	5′-TGGTAGTTGGAGCTGGTGGCGTAGGCA-3′
		Ras Rev		5′-GTCAACACCCTGTCTTGTCTTCGCTGAGGTC-3′
GBLP	P25388	GBLP Fw	416	5′-AGGTACACTGGGGTGGCTCTGCCTTGC-3′
		GBLP Rev		5′-TGGGTGTCTTGTGTCCGCTTCTCCCC-3′
Cyclophillin B	O88541	CPB Fw	518	5′-TTTTGCTGCCTGGACCCTCCGTGG-3′
		CPB Rev		5′-CACATCCTTCAGGGGCTTGTCCCGG-3′
TCP-1δ	P80315	TCP Fw	539	5-ATAGAAGCAGGAGATGGCACCACGTCGG-3′
		TCP Rev		5′-TCCTCTCGAAGCACTCGATCCATCTGGG-3′
β-Actin	P60710	ACT Fw	164	5′-CGCTCGTCGTCGACAACGGCTCC-3′
		ACT Rev		5′-CTTGCTCTGGGCCTCGTCGCCC-3′
GAPDH	P04797	GAPDH Fw	399	5′-TCAACGGCACAGTCAAGGCTGAGAATGG-3′
		GAPDH Rev		5′-ACAGTCTTCTGAGTGGCAGTGATGGCATGG-3′
FUS	P56959	FUS Fw	731	5′-TACAACCGAAGCAGTGGTGGCTATGAACCC-3′
		FUS Rev		5′-CTGCCACGTCGATCATCTCCATAGTTTCCC-3′

expression rate of each amplified gene. Amplification conditions were: 3 min at 94 °C, as initial step, then an amplification process of 30 s at 94 °C, 60 s at 60 °C, 60 s at 72 °C for 26–30 cycles and, finally, 8 min at 72 °C. The amplified products were resolved on a 1.5% agarose gel.

2.5. 2D-E and mass spectrometry analysis

The analysis was performed twice on nuclear extracts from three different batches of cell cultures. Samples containing 50–100 μg of total proteins were separated in a horizontal 2D-electrophoresis set-up Multiphor II (Amersham Biosciences, Milan, Italy) according to Gorg et al. [19] following heating at 95 °C for 6 min. IEF was performed by applying the samples at the cathodic side of IPG strips (0.5 mm × 170 mm), containing Immobilines NL 3–10, and running for 0.5 kV for 10 h and 3.5 kV for 9.5 h, at 10 °C. After equilibration, the IPG gel strips were transferred onto second-dimensional vertical gradient slab gels (9–16% T) and run with the Laemmli–SDS–discontinuous system. Protein detection was achieved using a sensitive ammoniacal silver stain [20]. Isoelectric points were estimated by the parallel running of a mixture of p*I* protein standards (BioRad, Richmond, CA, USA) and using identified internal proteins in the samples with known p*I*. Gels were scanned using a GS-710 Imaging Densitometer (BioRad, Richmond, CA, USA). 2D-E image computer analysis was carried out using the Melanie 2 software package [21]. Spots were detected and quantified automatically. Spot intensities were obtained in pixel units, normalized to the total absorbance of the gel and calculated as percentage relative volumes. The change index was defined as the ratio between the spot percentage relative volumes in transformed Ki-mol and normal FRTL-5 rat thyroid cell lines. All indexes were calculated based on the mean of the spot intensities of all six gels, with standard deviations. Significant differences in protein expression levels were determined by Student's *t*-test with a set value of $P < 0.05$.

Spots from 2D-E were excised from the gel, triturated and washed with water. Proteins were in-gel reduced, *S*-alkylated and digested with trypsin as described [22]. Digest aliquots were removed and used directly or subjected to a desalting/concentration step on μZipTipC_{18} (Millipore Corp., Bedford, MA, USA) before MALDI-TOF–MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic as matrix, and analysed by using Voyager-DE PRO and Voyager-DE STR mass spectrometers (Applied Biosystems, Framingham, MA, USA). PROWL software package was used to identify spots unambiguously from independent non-redundant sequence databases [23]. Candidates from peptide matching analysis were further evaluated by the comparison with their calculated molecular mass and p*I* using the experimental values obtained from 2D-E.

2.6. Recombinant protein expression and purification

Recombinant APE1/Ref-1 protein was obtained as a GST-tag fusion protein from over-expression in *E. coli* and then purified by GSH affinity chromatography from bacterial extracts as previously described [24]. Briefly, *E. coli* BL21 cell culture (2 ml), transformed with pGex-GST–APE1/Ref-1 or with pGex-GST alone, were grown overnight at 37 °C in the LB-medium supplemented with 50 μg/ml ampicillin. Fresh and pre-warmed (37 °C) LB medium was inoculated with the overnight cultures to an $O.D._{600}$ of 0.05–0.1. The cultures were grown until the $O.D._{600}$ reached a 0.5–0.7 value, at which time IPTG was added to a final concentration of 0.5 mM. Expression of GST and GST–APE1/Ref-1 fusion protein were induced by growing the cells for an additional 2 h at 37 °C. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, at 4 °C, resuspended in 10 ml of PBS, pH 7.4, per gram of bacterial pellet and lysed on ice by sonication. The lysate was centrifuged at 10,000 × *g* for 20 min, at 4 °C, and the GST and the GST–APE1/Ref-1 proteins were collected in the supernatant as soluble forms. Purification was performed by affinity chromatography using a glutathione-agarose resin (Amersham Biosciences, Milan, Italy) equilibrated in PBS containing 1% (v/v) Triton X-100. Following several washings with the same buffer, the recombinant proteins were eluted with 50 mM Tris–HCl, pH 7.5, containing 10 mM reduced glutathione. The collected samples were analysed by SDS–PAGE and silver staining. This procedure resulted in single protein bands with molecular mass of 30 kDa for GST and of roughly 66 kDa for the GST–APE1/Ref-1 fusion protein. Fractions containing purified proteins were stored at −80 °C.

2.7. GST-pulldown and immunoprecipitation assays

For GST-pulldown experiments, the purified GST and GST–APE1/Ref-1 recombinant proteins were used. For the interaction between the GST–APE1/Ref-1 and the FRTL-5 nuclear extract and between GST and the FRTL-5 nuclear extract, as a control, 1 μg of the former and 500 ng of the latter were mixed for 8 h at 4 °C with 100 μg of nuclear extracts. Then, co-precipitations were performed by addition of 30 μl of GST-agarose (Amersham Biosciences, Milan, Italy), washed three times with PBS in the presence of 0.1% NP-40 (w/v) and eluted with 50 μl of 10 mM reduced glutathione in PBS. Eluted samples were precipitated with 4 volumes of cold acetone, centrifuged and the pellets were washed with 200 μl of 20% (v/v) cold methanol. After centrifugation for 30 min at 12,000 × *g*, pellets were air-dried and dissolved in rehydrating solution for further 2D-E analysis, which was performed in the range pH 4.0–7.0, as described above.

2.8. Immunocytochemistry

Double immunostaining for the detection of β-actin and APE1/Ref-1 were done on FRTL-5 thyroid cells grown on slides. A monoclonal antibody to β-actin (Sigma–Aldrich Co., Milan, Italy) was used after antigen-retrieval microwave treatment of cultured cells in 0.01 M citrate buffer, pH 6.0. Slides were incubated with the primary antibody (10 μg/ml) for 1 h, at room temperature, followed by avidin–biotin complex alkaline phosphatase. Immunostaining for APE1/Ref-1

was performed with a specific 1:100 diluted monoclonal antibody [14], for 1 h, at room temperature, followed by avidin-biotin complex peroxidase (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The alkaline phosphatase activity was visualised with Fast blue BB-Naphtol AS (Sigma, Milan, Italy); peroxidase activity was visualised with 3,3′-diaminobenzidine-tetrahydrochloride as the chromogen. Control slides were immunostained under identical conditions, substituting the primary antibody with buffer solution.

3. Results

3.1. 2D-E analysis of nuclear extracts from Ki-mol and FRTL-5 cell lines

This work was undertaken to complete a previous one [14], devoted to identification of only qualitative differences occurring in nuclear proteome profiles of FRTL-5 and Ki-mol cell lines, by taking into consideration all the quantitative differences detectable. To investigate the presence of nuclear proteins differently expressed between the highly differentiated FRTL-5 rat thyroid cell line and the completely undifferentiated Ki-mol cell line, nuclear extracts were prepared as previously described [15]. The quality of nuclear extracts was tested for the presence of the nuclear-specific transcription factor Pax-8; the corresponding western blot analysis is reported in Fig. 1.

Then, proteomes of nuclear-enriched samples from FRTL-5 and Ki-mol cell lines were analysed by 2D-E. The analysis was performed twice on nuclear extracts from three different batches of cell cultures and silver staining allowed comparing a number of 100–150 spots from cell lysates. Samples normalization was performed by assaying protein content using SDS–PAGE or IEF. The differences present in the region of the gel corresponding to components with a molecular mass of 120-15 kDa are displayed in Supplementary Fig. 1 (panel A and B). For the purpose of this study, differently expressed proteins were considered those detectable in all stained gels and showing a difference greater than three-fold variation in spot relative intensity volume between the two comparison groups. To sum up with previous study [14], thirteen proteins were identified as being differently expressed between the two cell lines (indicated with P), and thirteen were identified as commonly represented species (indicated with C). Among the differential proteins, P0, P1, P2 and P3 were specifically up-regulated in FRTL-5 cells, while the remaining nine were up-regulated in the Ki-mol cell line. The corresponding spots from two replicas of two batches of cell cultures were alkylated, digested with trypsin and analysed by MALDI-TOF–MS. Peptide mass fingerprint analysis allowed their unambiguous identification as reported in Table 2. The common protein species, indicated with the letter C, have been listed for completeness. Besides a number of differentially expressed proteins clearly belonging to the nuclear compartment, a series of equally represented protein species (e.g. aconitate hydratase, isocitrate dehydrogenase, citrate synthase, etc.) are specific of the cytoplasmic or the mitochondrial compartments and thus represents contaminations. This result could be ascribed to the difficulty of obtaining completely homogeneous purified nuclear compartments and to the high abundance of these proteins within cells.

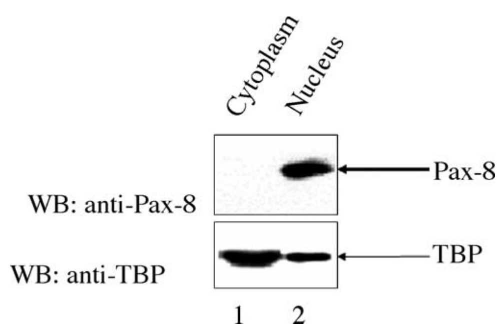

Fig. 1. Immunoblotting of subcellular fractions of FRTL-5 thyroid cells with anti-Pax8 antibody. Thirty micrograms of cytoplasmic (lane 1) and nuclear (lane 2) fractions of FRTL-5 cells were run onto a 12% SDS–PAGE. After blotting onto a nitrocellulose membrane, the filter was probed with the nuclear specific αPax8-187 antibody followed by peroxidase conjugated goat anti-rabbit IgG. Bands were visualized by ECL chemiluminiscent substrate. As a loading control the presence of the TATA binding protein (TBP) was also tested by using an anti-TBP antibody.

3.2. Analysis of the nuclear expression and gene expression of some of the identified proteins in normal and transformed thyroid cell lines

To validate the data obtained by 2D-E analysis, western blot analysis for some of the identified proteins were performed on nuclear extracts of FRTL-5 and Ki-mol cell lines; the corresponding results are reported in Fig. 2(panel A) . Thirty micrograms of nuclear proteins from both cell lines were separated by 12% SDS–PAGE, blotted onto a nitrocellulose membrane and analysed with the specific antibodies raised against Gal-3, APE1/Ref-1, Gal-1 and EF-1α protein. Gal-3 and Gal-1 were confirmed to be qualitatively expressed only in the nuclear compartment of the transformed cell line, while no expression was detectable in the normal FRTL-5 cell line, as previously demonstrated [14]. On the other hand, APE1/Ref-1 was found quantitatively increased in the nuclear fraction of the normal cell line with respect to the transformed one, while EF-1α resulted increased in the transformed cell line.

Differential proteomic analysis on nuclear enriched samples may provide information not only on differences occurring on gene expression but also on additional control mechanisms of the identified proteins. To better address this hypothesis in the system under investigation, we performed RT-PCR analysis on 10 genes identified by proteomic analysis (see Fig. 2, panel B). For three of these (i.e. TCP-1δ, β-actin and GAPDH) that were equally represented in the nuclear enriched samples of the two thyroid cell lines, RT-PCR analysis was confirmatory of proteomic data. Similarly, for the Ki-ras gene (up-regulated in Ki-mol cells) and cyclophilin B (up-regulated in FRTL-5 cells), RT-PCR analysis was also confirmatory of the proteomic data. However, for the remainder genes analyzed by RT-PCR (i.e. RAN, EF-1α, FUSE-BP, FUS, GBLP), the mRNA level did not

Table 2
Identified proteins and relative expression in the FRTL-5 and Ki-mol cells

Spot no.	Protein	Sequence coverage (%)	SwissProt entry	MW	p*I*	Change index	Biological function
P0	Erp29	22	P52555	26996	6.08	0.001	ER molecular chaperone
P1	Aminoacylase 1 (Acy 1)	25	Q9CR15	40562	6.17	0.004	Proteolytic enzyme
P2	APE1/Ref-1	36	Q99PF3	34978	6.97	0.187	Redox coactivator, BER enzyme
P3	Peptidyl-prolyl cis-trans isomerase (Cyclophylin B)	39	O88541	12410	7.52	0.001	Peptidylprolyl isomerase
P4	Far upstream element binding protein 1 (FUSE BP)	25	Q12828	65848	6.69	34.235	ssDNA-binding protein regulating Myc expression
P5	RNA-binding protein FUS	31	P56959	55979	7.35	236.333	ss- and ds-DNA binding protein
P6	Elongation factor 1-α (EF-1α)	59	Q64718	43211	7.34	4.879	Protein neosynthesis
P7	Guanine nucleotide-binding protein β subunit 2-like 1 (GBLP)	35	P25388	30111	6.76	5.582	PKC binding
P8	Galectin 3	46	P08699	27521	6.87	291.285	Beta galactoside binding lectin [14]
P9	GTP-binding nuclear protein Ran	24	P62827	23778	6.63	67.593	GTP-binding protein involved in nucleocytoplasmic transport and control of cell cycle
P10	Transforming protein p21, Ki-Ras	17	P46203	20429	6.56	26.569	Small GTPase involved in cell proliferation
P11	Arpc5	37	Q9CPW4	15685	5.43	152.833	Unknown
P12	Galectin 1	49	P11762	12111	5.14	213.666	Beta galactoside binding lectin [14]
[5pt] C1	78 kDa glucose-regulated protein	33	P06761	70169	5.20	0.571	ER molecular chaperone
C2	Aconitate hydratase	39	Q9ER34	96620	6.80	0.951	Metabolic pathways
C3	Transketolase	29	P50137	65310	6.77	0.948	Metabolic pathways
C4	T-complex protein 1, δ subunit	41	P80315	53299	6.90	0.668	Molecular chaperone
C5	NADP+-specific isocitrate dehydrogenase	40	Q9EQK1	40775	7.01	0.584	Metabolic pathways
C6	L-Lactate dehydrogenase A chain	27	P04642	34076	6.98	0.884	Metabolic pathways
C7	Citrate synthase	21	Q9CZU6	40549	6.87	0.570	Metabolic pathways
C8	β-Actin	65	P60710	42000	5.25	1.716	Cytoskeleton
C9	Fructose-bisphosphate aldolase A	44	P05065	38212	6.86	0.614	Metabolic pathways
C10	Glyceraldehyde-3-phosphate dehydrogenase	57	P04797	35557	6.79	1.283	Metabolic pathways
C11	Malate dehydrogenase	26	P04636	34841	7.04	0.636	Metabolic pathways
C12	Phosphoglycerate mutase	20	P25113	26683	6.55	0.608	Metabolic pathways
C13	hnRNP A1	25	P04256	22040	6.82	0.676	RNA-binding protein

The change index reports the ratio of spot intensities (relative volumes) for transformed Ki-mol rat thyroid cell lines with respect to normal FRTL-5. These values were calculated based on the mean of the spot intensities of each six gels. Only indexes with a statistical significance at the $P < 0.05$ level of probability were reported.

parallel the data obtained by differential proteomic analysis of the nuclear compartment. Therefore, these data strongly suggest that for a significant number of the identified proteins, the difference existing between the two cell systems may be not due to an altered gene expression but, rather, to alterations of other control mechanisms, such as translation efficiency and/or subcellular localization.

3.3. APE1/Ref-1 interacting partners

APE1/Ref-1 is a dual function protein involved both in the base excision repair (BER) pathways of DNA lesions and in eukaryotic transcriptional regulation of gene expression as a redox co-activator of different transcription factors in different cell systems [9]. Though its main subcellular localization is

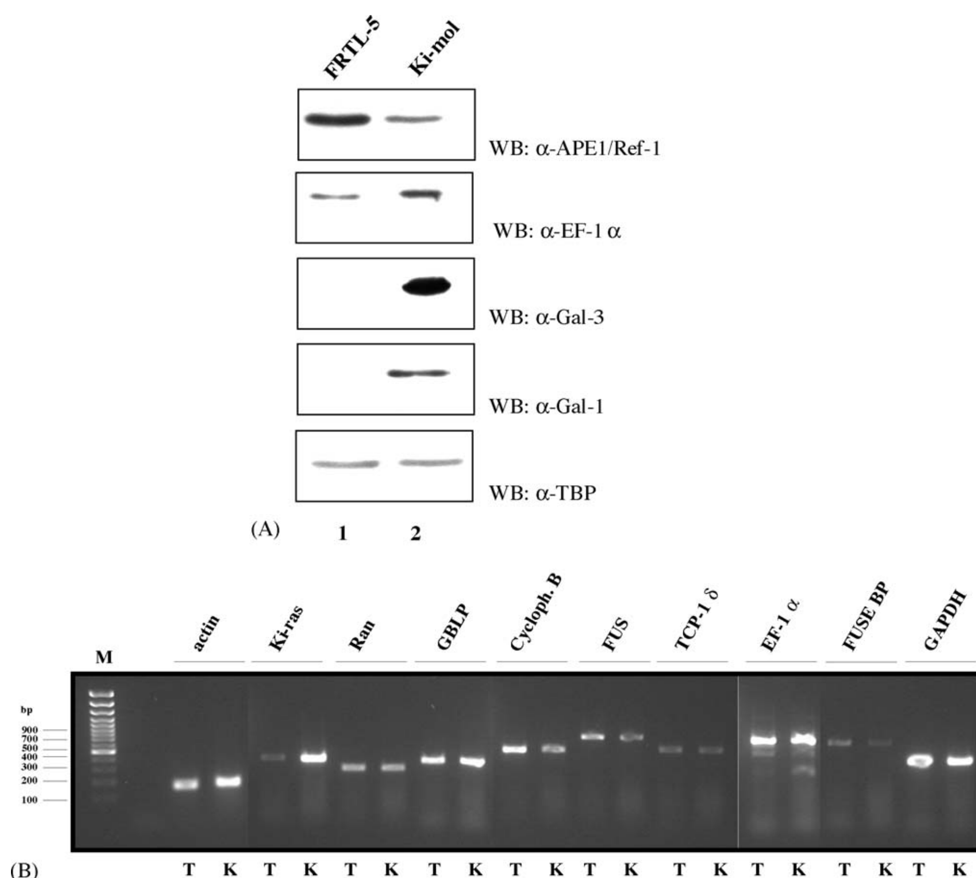

Fig. 2. Western blot analysis and RT-PCR analysis of some of the proteins/genes identified by proteomic analysis in normal FRTL-5 and transformed Ki-mol rat thyroid cell lines. Panel A. Western blot analysis of Gal-1, Gal-3, APE1/Ref-1 and EF-1α in FRTL-5 and Ki-mol thyroid cells. Thirty micrograms of nuclear extracts from FRTL-5 and Ki-mol cells were separated onto a 12% SDS–PAGE and analysed for the presence of Gal-1, Gal-3, APE1/Ref-1 and EF-1α proteins. After blotting onto a nitrocellulose membrane, the filter was probed with the specific antibodies followed by peroxidase conjugated secondary antibody. Bands were visualized by ECL chemiluminiscent substrate. As a loading control the presence of actin was also tested by using an anti-TBP polyclonal antibody. Panel B. RT-PCR analysis of some of the genes identified by proteomic analysis in FRTL-5 (T) and Ki-mol (K) cell lines. Representative agarose gel electrophoresis of PCR products, see Materials and Methods for further details. M, size markers: 100 bp ladder (Gibco, BRL), appropriate sizes are indicate. For each gene amplification, a proper negative control containing not retro-transcribed mRNA instead of template cDNA was also included but it is not reported in this figure for space reasons. The figure is representative of three independent experiments.

nuclear, cytoplasmic localization was also described in several human thyroid tumors [9]. In particular, immunohistochemical analysis of thyroid carcinomas showed a strong APE1/Ref-1 cytoplasmic staining [25] and the derived cell lines showed a reduction in the nuclear/cytoplasmic ratio of the protein levels [26], with respect to non-tumoral cells. These and other evidences suggest that APE1/Ref-1 may possibly be involved in the tumorigenic process of thyroid carcinoma. Moreover, we have already demonstrated that APE1/Ref-1 is able to control TFC-specific transcription factors activity by redox mechanisms [9]. Therefore, we tried to explore the possibility to study the interacting partners of APE1/Ref-1 in the nuclear compartment of FRTL-5 thyroid cells. To this end, we used a GST-pulldown approach by means of recombinant proteins as bait. APE/Ref-1 protein was expressed as a GST-fusion product with high yield and purity, as reported in Fig. 3 , panel A and B. For the interaction between the GST–APE1/Ref-1 and the FRTL-5 nuclear extract and between GST and the FRTL-5 nuclear extract (control), proteins were mixed with nuclear extracts. Then, co-precipitation was performed by addition of GST-agarose, followed by extensive washing and suspension. GST-pulldown materials from control and APE1/Ref-1 co-precipitation experiments were separated by 2D-E and silver stained. Differential analysis showed the presence of a specific spot only in the GST–APE1/Ref-1 pulldown sample that was subsequently identified as nuclear β-actin.

The functional relevance of β-actin nuclear localization over APE1/Ref-1 function requires that both proteins have to reside in the same cellular compartment. To test this hypothesis, we performed co-localization study by immunocytochemistry in FRTL-5 thyroid cells. As expected, a clear co-localization of β-actin and APE1/Ref-1 occurred in the cell system used for this study (Fig. 3, panel D). To definitively prove the occurrence of APE1/Ref-1/β-actin interaction, circumstantiating its functional role in thyroid cells, a co-immunoprecipitation approach was also used (Fig. 3, panel E). FRTL-5 cells were stimulated for 10 min with 1 mM H_2O_2 and then nuclear extracts were prepared. FRTL-5 cells nuclear extracts were treated with protein A and anti-APE1/Ref-1 antibody [17] to recover the APE1/Ref-1-immunoprecipitated material. After extensive washings of the

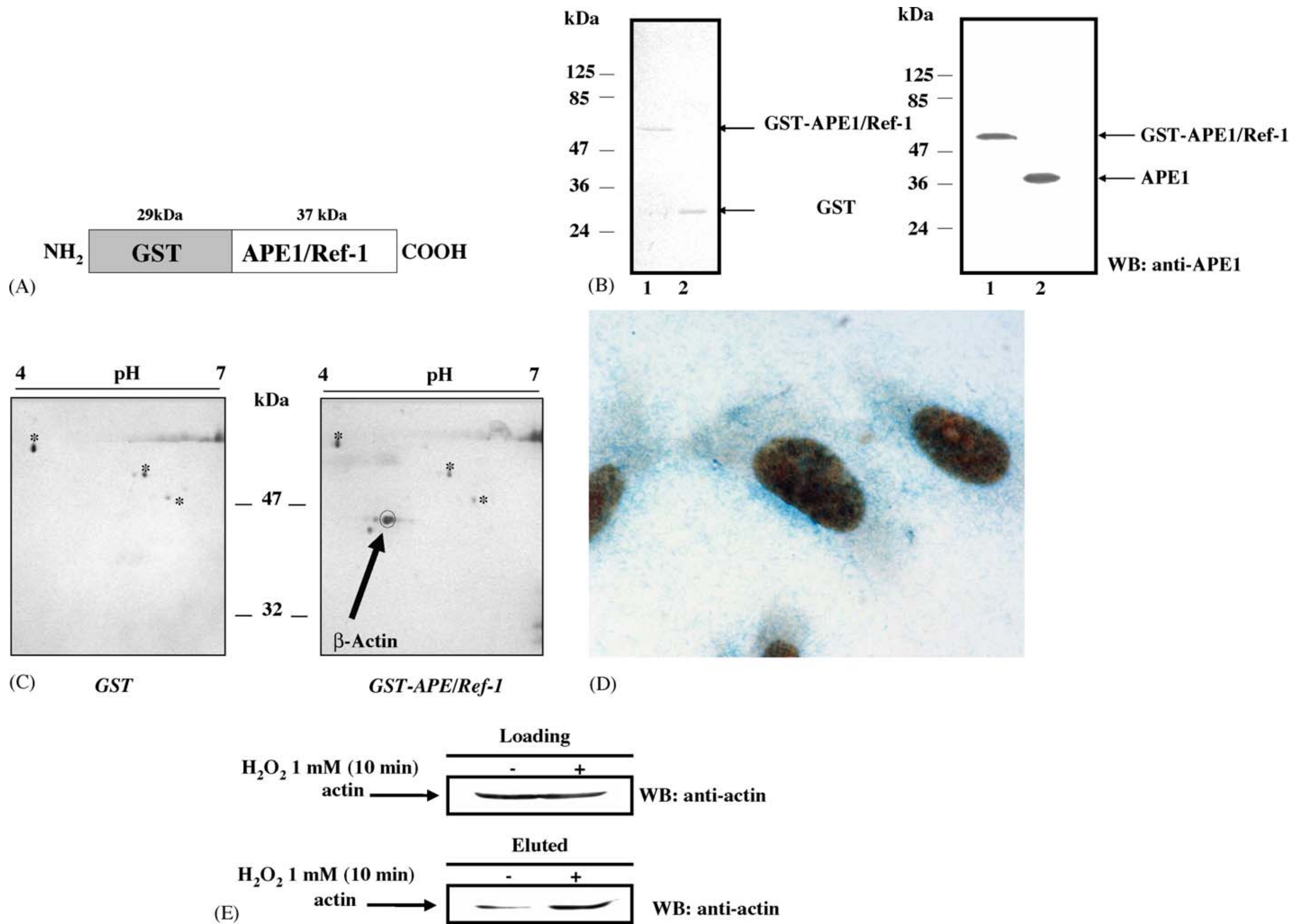

Fig. 3. Recombinant and endogenous APE1/Ref-1 specifically interact with nuclear actin. (Panel A) Domain organization of the GST–APE1/Ref-1 fusion protein. (Panel B) Purification of the GST–APE1/Ref-1 fusion protein. (Left) Silver staining: lane 1, purified GST–APE1/Ref-1 recombinant protein from BL21 bacterial cells; lane 2, purified GST. (Right) Western blot analysis of the recombinant GST-APE/Ref-1 (lane 1) and endogenous APE1/Ref-1 from nuclear extracts of FRTL-5 cells (lane 2), performed with the anti-APE1/Ref-1 specific antibody. (Panel C) GST-Pulldown coupled to 2D-E analysis and MALDI-TOF–MS identification of APE1/Ref-1 interacting partners. APE1/Ref-1 directly interacts with nuclear β-actin. GST-Pulldown assay by 500 ng of purified GST or 1 μg of purified GST–APE1/Ref-1 were incubated with 100 μg of nuclear extracts of FRTL-5 cells. Then, the mixtures were trapped by GST-agarose followed by three washings in PBS, 0.1% (w/v) NP-40 and eluted by addition of 10 mM reduced glutathione in PBS. Eluted samples were precipitated by acetone and further analysed by 2D-E analysis coupled to MALDI-TOF–MS identification. Position of β-actin is indicated. *Indicates equally represented protein spots. (Panel D) Endogenous APE1/Ref-1 and β-actin colocalize in FRTL-5 cells. FRTL-5 cells were assayed by immunocytochemistry. Detection of APE1/Ref-1 was performed with the specific monoclonal antibody followed by streptavidin-biotin complex peroxidase, while β-actin was detected by specific monoclonal antibody (Sigma, Milan, Italy) followed by streptavidin-biotin complex alkaline phosphatase, as described in the experimental section. Alkaline phosphatase activity was visualised with Fast blue BB-Naphtol AS; peroxidase activity was visualised with 3,3′-diaminobenzidine-tetrahydrochloride as the chromogen. Control slides were immunostained under identical conditions, substituting the primary antibody with buffer solution (not shown). (Panel E) Nuclear β-actin directly interacts with endogenous APE1/Ref-1. Co-immunoprecipitation assay of endogenous APE1/Ref-1 and β-actin by the specific anti-APE1/Ref-1 antibody. FRTL-5 cells were stimulated for 10 min with 1 mM H_2O_2 and then nuclear extract were prepared. FRTL-5 cells nuclear extracts were added with protein A and anti-APE1/Ref-1 antibody following gentle shaking at 4 °C, for 2 h, and spinning at 12,000 rpm for 3 min to pellet and recover the APE1/Ref-1-immunoprecipitated material. After three washings of the pellets with 200 μl PBS immunoprecipitated materials were eluted by directly boiling in 50 μl of Laemmli sample buffer and further analysed by western blotting for the presence of β-actin. Twenty microliters of each eluted samples were separated onto a 12% SDS–PAGE and further analysed by western blotting with antibodies specific for β-actin protein. The equal amount of β-actin in the nuclear compartment of unstimulated and stimulated FRTL-5 cells before immunoprecipitation was also analysed as a control (loading).

pellets, immunoprecipitated materials were eluted and further analysed by western blotting for the presence of β-actin. As it was evident, H_2O_2 stimulation promoted a significant increase of APE1/Ref-1/β-actin interaction (Fig. 3, panel E). These data demonstrated that interaction between APE1/Ref-1 and β-actin occurs in vivo in the nuclear compartment of FRTL-5 thyroid cells and this interaction may be modulated by changes in the cellular redox status.

4. Discussion

Disregulation in the differentiation process is the main cause of thyroid tumorigenesis [27]. Control of the differentiation process is largely performed at the transcriptional level in the nuclear compartment. Therefore, we applied a differential proteomic approach on nuclear-enriched extracts from normal rat thyroid cell line FRTL-5 compared to transformed Ki-mol cell

line, to identify possible markers of dedifferentiation in thyroid cells. Four proteins were specifically up-regulated in the FRTL-5 cell line while nine proteins resulted specifically increased in the nuclear compartment of the transformed cell line (Table 1). RT-PCR analysis on gene expression of eight of these proteins suggested that altered regulation occurring to a significant number of these (5/8) could be eventually ascribable to a post-transcriptional/post-translational level.

Among molecular species whose expression was significantly higher in normal thyroid cells, a proteolytic enzyme was detected, namely aminoacylase 1 (Acy1). This protein was previously found as associated to p53 expression in thyroid cells [28]. Our findings, paralleling recent proteomic investigations that demonstrated a decreased protein expression in several different tumorigenic models [29–32], underline the relevance of this gene as a good new candidate for cancer therapy. Similarly, two chaperone proteins, i.e. Erp29 and cychlophylin B, were found more significantly expressed in the normal thyroid cell line, thus reinforcing the recent hypothesis that also this class of proteins could play a relevant role in the tumorigenic process [33–35].

Among the nuclear proteins specifically up-regulated in the normal cell line, we identified the multifunctional protein APE1/Ref-1 [9]. APE1/Ref-1 is regulated at both the transcriptional and post-translational level. In terms of transcriptional regulation, the effects of the so-called Reactive Oxygen Species (such as H_2O_2, O_2^- and $OH^\bullet$ and collectively indicated as ROS) on APE1/Ref-1 induction have been the most intensively studied. Data reported so far demonstrated that oxidative agents, such as H_2O_2, and ROS-generating injuries such as UV-radiation, promote a transient APE1/Ref-1 protein and mRNA induction which correlates with an increase of its endonuclease and redox activities. The post-translational regulation of APE1/Ref-1 activities seems to reside into two non-mutually exclusive mechanisms, i.e. subcellular localization and post-translational modification degree. On one side, APE1/Ref-1 undergoes an active cytoplasm to nucleus translocation in different cells upon ROS exposure [25,36]. Due to the importance APE1/Ref-1 plays in thyroid tumorigenesis [9], we concentrated on identifying nuclear interacting protein partners in FRTL-5 thyroid cells. Nuclear β-actin resulted physically associated to APE1/Ref-1 and the nature of this interaction was enhanced by exposing cells to H_2O_2 for short times. The biological relevance of this interaction is, at the moment, completely unknown. However, it is becoming increasingly evident that β-actin plays several functional roles in the nuclear compartment of eukaryotic cells, particularly in the control of gene expression by binding to RNA Pol I [37]. The discovery that actin is a component of the mammalian chromatin-remodelling BRG-associated factor (BAF) complex [38], together with previous evidences indicating a direct association of actin with RNA Pol II in the control of transcription initiation [39,40], have spurred a renewed interest in the functions of nuclear actin. Additional experiments are now being performed to address the issue of whether β-actin association plays a functional role in controlling APE1/Ref-1 specific functions and whether this association is affected in thyroid tumorigenesis.

On the other side, among proteins specifically up-regulated in the transformed cell line, a number of proteins involved in the control of nuclear specific processes such as proliferation-associated gene expression (i.e. FUSE BP, FUS) or nuclear transport (i.e. Ran) were found. Interestingly, the FUSE BP binds in vivo and in vitro with the single-stranded far upstream element (FUSE) located upstream of the *c*-myc proto-oncogene [41] and regulates its expression levels by controlling cell proliferation. This evidence, deserving further studies, represents the very first evidence, in the thyroid cell model, for an involvement of this protein in the tumorigenic process.

In addition, we found EF-1α as specifically upregulated in the transformed cell line. This finding parallels a growing body of evidences suggesting the involvement of translation elongation factors (EFs) at the onset of oncogenesis [42].

In conclusion, here, we showed a simple approach aimed to the identification of nuclear factors involved in the control of cell differentiation. The choice of inspecting nuclear enriched fractions, rather than using total cell extracts, gave the possibility to study protein species that could be involved in control of transcriptional processes, even if our approach was unable to show any transcription factor. This was due to the particular approach used. In fact, to study alterations involving very low expressed proteins such as transcription factors (10–100 copies/cell), it could have been necessary to use affinity chromatography strategies, such as DNA-cellulose resins. Nevertheless, our approach allowed to significantly reduce the whole cell proteome and improved the knowledge of putative candidate proteins possibly involved in the control of differentiation and potential markers of the thyroid tumorigenic process. Further expression analysis of the identified proteins in human thyroid tumors will test this hypothesis.

Acknowledgments

This work was supported by grants from the Ministero per l'Università e la Ricerca Scientifica e Tecnologica (MIUR) to G.D. and G.T. (FIRB 2003 RBNE0155LB-003) and A.S. (FIRB2001 RBAU01PRLA-002), from National Research Council to A.S. (AG-P01-ISPAAM-C2) and from the University of Udine to G.T.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2005.12.025.

References

[1] S. Hanash, Nat. Rev. 4 (2004) 638.
[2] P. Oh, Y. Li, J. Yu, E. Durr, K.M. Krasinska, L.A. Carver, J.E. Testa, J.E. Schnitzer, Nature 429 (2004) 629.
[3] M.J. Page, B. Amess, R.R. Townsend, R. Parekh, A. Herath, L. Brusten, M.J. Zvelebil, R.C. Stein, M.D. Waterfield, S.C. Davies, M.J. O'Hare, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 12589.
[4] K. Struhl, Cell 98 (1999) 1.
[5] I. Herskowitz, Nature 342 (1989) 749.
[6] B.M. Spiegelman, R. Heinrich, Cell 119 (2004) 157.

[7] A.J. Berk, Biochim. Biophys. Acta 1009 (1989) 103.
[8] G. Damante, G. Tell, R. Di Lauro, Prog. Nucl. Acids Res. 66 (2001) 307.
[9] G. Tell, G. Damante, D. Caldwell, M.R. Kelley, Antioxid. Red Signal 7 (2005) 367.
[10] A. Fusco, M.T. Berlingieri, P.P. Di Fiore, G. Portella, M. Greco, G. Vecchio, Mol. Cell Biol. 7 (1987) 3365.
[11] L. Perrone, G. Tell, R. Di Lauro, J. Biol. Chem. 274 (1999) 4640.
[12] L. Aurisicchio, R. Di Lauro, M. Tannini, J. Biol. Chem. 273 (1998) 1477.
[13] F.S. Ambesi-Impiombato, L.A. Parks, H.G. Coon, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 3455.
[14] I. Paron, A. Scaloni, A. Pines, A. Bachi, F.T. Liu, C. Puppin, M. Pandolfi, L. Ledda, C. Di Loreto, G. Damante, G. Tell, Biochem. Biophys. Res. Commun. 302 (2003) 545.
[15] B. Thiede, C. Dimmler, F. Siejak, T. Rudel, J. Biol. Chem. 276 (2001) 26044.
[16] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[17] Y. Xu, D. Moore, J. Broshears, L. Liu, T.M. Wilson, M.R. Kelley, Anticancer Res. 37 (1997) 13.
[18] D.H. Moore, H. Michael, R. Tritt, S.H. Parsons, M.R. Kelley, Clin. Cancer Res. 6 (2000) 602.
[19] A. Gorg, W. Postel, S. Gunther, Electrophoresis 9 (1988) 531.
[20] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Anal. Chem. 68 (1996) 850.
[21] R.D. Appel, D.F. Hochstrasser, Methods Mol. Biol. 112 (1999) 363.
[22] F. Talamo, C. D'Ambrosio, S. Arena, P. Del Vecchio, L. Ledda, G. Zehender, L. Ferrara, A. Scaloni, Proteomics 3 (2003) 440.
[23] W. Zhang, B.T. Chait, Anal. Chem. 72 (2000) 2482.
[24] I. Huq, T.M. Wilson, M.R. Kelley, W.A. Deutsch, Mutat. Res. 337 (1995) 191.
[25] G. Tell, L. Pellizzari, C. Pucillo, F. Puglisi, D. Cesselli, M.R. Kelley, C. Di Loreto, G. Damante, J. Mol. Endocrinol. 24 (2000) 383.
[26] D. Russo, F. Arturi, S. Bulotta, L. Pellizzari, S. Filetti, G. Manzini, G. Damante, G. Tell, J. Endocrinol. Invest. 24 (2001) RC10.
[27] C.E. Hedinger, Schweiz. Med. Wochenschr. 123 (1993) 1673.
[28] I. Paron, C. D'Ambrosio, A. Scaloni, M.T. Berlingieri, P.L. Pallante, A. Fusco, N. Bivi, G. Tell, G. Damante, J. Mol. Endocrinol. 34 (2005) 199.
[29] W. Ying, K. Zhang, X. Qian, L. Xie, J. Wang, X. Xiang, Y. Cai, D. Wu, Proteomics 3 (2003) 64.
[30] S. Balabanov, U. Zimmermann, C. Protzel, C. Scharf, K.J. Klebingat, R. Walther, Eur. J. Biochem. 268 (2001) 5977.
[31] A. Scaloni, W. Jones, M. Pospischil, S. Sassa, O. Schneewind, A.M. Popowicz, F. Bossa, S.L. Graziano, J.M. Manning, J. Lab. Clin. Med. 120 (1992) 546.
[32] W.M. Jones, A. Scaloni, F. Bossa, A.M. Popowicz, O. Schneewind, J.M. Manning, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 2194.
[33] J.K. Myung, L. Afjehi-Sadat, M. Felizardo-Cabatic, I. Slavc, G. Lubec, Proteome Sci. 2 (2004) 8.
[34] S. Gu, Z. Liu, S. Pan, Z. Jiang, H. Lu, O. Amit, E.M. Bradbury, C.A. Hu, X. Chen, Mol. Cell Proteomics 3 (2004) 998.
[35] L. Whitesell, S.L. Lindquist, Nat. Rev. Cancer 5 (2005) 761.
[36] G. Tell, A. Zecca, L. Pellizzari, P. Spessotto, A. Colombatti, M.R. Kelley, G. Damante, C. Pucillo, Nucleic Acids Res. 28 (2000) 1099.
[37] V.V. Philimonenko, J. Zhao, S. Iben, H. Dingova, K. Kysela, M. Kahle, H. Zentgraf, W.A. Hofmann, P. de Lanerolle, P. Hozak, I. Grummt, Nat. Cell Biol. 6 (2004) 1165.
[38] K. Zhao, W. Wang, O.J. Rando, Y. Xue, K. Swiderek, A. Kuo, G.R., Cell 625 (1998) 95.
[39] J.M. Egly, N.G. Miyamoto, V. Moncollin, P. Chambon, EMBO J. 3 (1984) 2363.
[40] S.S. Smith, K.H. Kelly, B.M. Jockusch, Biochem. Biophys. Res. Commun. 86 (1979) 161.
[41] R. Duncan, L. Bazar, G. Michelotti, T. Tomonaga, H. Krutzsch, M. Avigan, D. Levens, Genes Dev. 8 (1994) 465.
[42] M. Al-Maghrebi, J.T. Anim, A.A. Olalu, Anticancer Res. 25 (2005) 2573.