Identification of Genes Downregulated in Tumor Cells Expressing Antisense Glutaminase mRNA by Differential Display

Mercedes Martín-Rufián
Juan A. Segura
Carolina Lobo
José M. Matés
Javier Márquez*
Francisco J. Alonso*

Deparmento de Biología Molecular y Bioquímica; Facultad de Ciencias; Universidad de Málaga; Málaga, Spain

ABSTRACT

Ehrlich ascites tumor cells (EATC) is a highly proliferative malignant cell line derived from mouse mammary epithelia, whereas their derivative, 0.28AS-2 cells, expressing antisense glutaminase mRNA, show a less transformed phenotype and loss of their tumorigenic capacity in vivo correlated with an inhibition of glutaminase expression. The mRNA differential display technique was applied to these two cell lines for the identification and isolation of genes whose transcription was altered. Side-by-side comparisons of cDNA patterns among relevant RNA samples revealed four genes significantly downregulated in 0.28AS-2 cells: high-mobility group Hmga2 protein, Fmnl3 or formin-like protein 3, Nedd-4 ubiquitin-protein ligase, and ubiquitin carboxyl-terminal hydrolase Usp-15. These positives were confirmed by Northern analysis. The four targeted genes have relevant functions in cell growth and proliferation. Our results show the validity of mRNA differential display technique to get insights into the molecular mechanisms underlying the acquisition of a more differentiated phenotype by tumor cells after inhibition of glutaminase expression.

INTRODUCTION

Glutaminase (EC 3.5.1.2) has a critical role in tumors and rapidly dividing cells and its activity has been correlated with malignancy. 1 To gain insights into the role of this enzyme in tumor cells, we previously hypothesized that selective inhibition of glutaminase expression may decrease proliferation rate and malignancy in cancer cells. Ehrlich ascites tumor cells (EATC) were stably transfected with an antisense cDNA construct of rat kidney glutaminase. The transfected cells, named 0.28AS-2, showed reversion of the transformed phenotype in vitro and lost their tumorigenic capacity in vivo when inoculated in isologous mice. 2

Some phenotypic changes have been previously detected in 0.28AS-2 cells compared with the wild-type EATC cells. For example, expression of both epithelial mucin-1 (MUC-1) and N-acetyl-α-D-galactosaminidase, proteins implicated in tumor's escape from the host immune system, were markedly diminished in 0.28AS-2 cells. 3 These changes were related to an effective anti-tumor immune response, which makes 0.28AS-2 cells unable to grow in the peritoneal cavity of mice and develop tumors in vivo. Furthermore, cells expressing antisense glutaminase mRNA contain lower levels of glutathione and higher number of apoptotic cells than wild-type EATC cells. 4

Since the technique of differential display (DD) was first described by Liang and Pardee, 5 a great number of studies have followed this method to distinguish mRNA populations in comparative studies. Although in the last years the use of DNA microarrays has gained favor, DD still remains as a highly popular technique. This could be explained by several advantages that make DD very suitable for massive analysis of differential expression of mRNAs: the lower amount of total RNA required, the possibility of detecting novel genes and transcripts, and the lower costs. 6 Furthermore, serious flaws have been recently revealed for microarrays regarding lack of reproducibility when the same RNA sample is analyzed with arrays from different manufacturers. 7 In this work, we have implemented the differential display technique to compare gene expression patterns in EATC and 0.28AS-2 tumor cell lines. The aim of the study is to identify, isolate and characterize genes responsible of changes associated with the reversion of the malignant phenotype.

MATERIALS AND METHODS

Cell cultures. The EATC line, from the American Type Culture Collection, and its derivative 0.28AS-2 were grown in RPMI (Sigma) medium supplemented with 10% FCS and antibiotics. Cultures were incubated in a humidified atmosphere at 37°C with 5% CO2/95% air. The transfected
cell line 0.28AS-2 was obtained by lipofection of EATC cells with the plasmid pCDNA3 containing an anti-sense 3' cDNA segment (0.28 kb) of rat kidney GA, as previously described. Transfected cells showed a marked decrease in GA activity and a very low level of GA protein expression compared with controls.

Isolation of RNA. Total RNA was isolated from EATC and 0.28AS-2 cells using the RNAqueous-4PCR kit (Ambion) following the manufacturer's instructions. The concentration and purity of RNA samples were determined by UV absorbance spectrophotometry and electrophoresis.

Differential display RT-PCR. Differential display was performed using the Delta Differential Display Kit (Clontech Laboratories) according to the manufacturer's instructions. DNA-free total RNA (2 µg) isolated from EATC and 0.28AS-2 cells was reverse-transcribed in 10 µl of RT buffer (50 mM Tris, 6 mM MgCl₂, 75 mM KCl, pH 8.3) containing 1 mM each of dATP, dCTP, dGTP and dTTP, 1.2 µCi [α-32P]dCTP (3000 Ci/mmol) and 200 units of MMLV-reverse transcriptase. The samples were incubated at 42°C for 60 min, then at 75°C for 10 min to inactivate the reverse transcriptase. Each cDNA sample obtained was diluted 1:10 (dilution A) and 1:40 (dilution B) and stored at -20°C for subsequent PCR reactions.

Amplification of cDNA fragments was performed in 20 µl PCR reactions, each in the presence of 1 of the 90 possible combinations of arbitrary upstream (designated as “P”) and downstream (designated as “T”) primers supplied by the manufacturer. The sequences of P and T primers used in these reactions are shown in Table 1. Each reaction mixture contained 1 µl first-strand cDNA, 1 µl PCR buffer (40 mM Tricine-KOH, pH 9.2, 15 mM KOAc, 3.5 mM Mg(OAc)₂, BSA 3.75 mg/ml), 50 µM dNTPs, 1.2 µCi [α-32P]dATP (Hartmann Analytic GmbH), 1 µM of P primer, 1 µM of T primer and 0.4 µl 50× Advantage cDNA polymerase mix (BD Biosciences Clontech). Reactions were carried out in a DNA Thermocycler iCycler (Bio-Rad) with the following parameters: 1 cycle at 94°C for 5 min, 40°C for 5 min, 68°C for 5 min; 2 cycles at 94°C for 2 min, 40°C for 5 min, 68°C for 5 min; 25 cycles at 94°C for 1 min, 60°C for 1 min, 68°C for 2 min, and a final elongation step at 68°C for 7 min. Control reactions were set up using sterile nuclease-free water or each DNase-treated RNA instead of the cDNA solution.

PCR products obtained from dilutions A and B of each first-strand cDNA produced from RNA of EATC and 0.28AS-2 cells were size-fractionated in parallel by denaturing electrophoresis in 6% polyacrylamide/8 M urea gels using a Genomyx-LR DNA Sequencer (Genomyx). Bands of interest were excised from the gels, eluted, reamplified by PCR, and then used as probes for Northern blot.

Elution and reamplification of DNA Fragments. To elute the amplified DNA fragments from the gel, portions of the polyacrylamide gel were cut and rehydrated in 40 µl of elution buffer (2x PCR buffer) for 90 min at 94°C. After centrifugation, the DNA present in the supernatant was collected. DNA fragments were reamplified using the same primer set and PCR conditions described above, except that no radio-labelled dATP was added. A small amount of the sample was run on an agarose gel and stained with ethidium bromide. The remaining samples were stored at -20°C for subcloning.

Cloning and sequencing of cDNA fragments. Reamplified cDNA fragments were cloned into pGem-T Easy vector system (Promega). Both strands of the cloned cDNA fragment were sequenced in both directions using M13 reverse (-24) primer and M13 forward (-20) primer on an automated DNA Sequencer (ABI PRISM 3700) from Applied Biosystem with the manufacturer’s own protocol and reagents. Sequencing was performed in the sequencing core facility of the Centro de Investigaciones Biológicas (CIB, Madrid). Nucleotide sequence homology search analyses of the Ensemble and GenBank databases were performed using the BLAST program. The isolated clones from differential display were used as probes, except for Fasl (GenBank accession no. BC058663) and Eif 5 (GenBank accession no. BC039275) genes whose cDNA clones were purchased from the I.M.A.G.E. Consortium. They were then used to isolate cDNA probes suitable for Northern analysis. Equal loading of the gels was assessed by ethidium staining of ribosomal RNAs. Specific signals on the filters were normalized by using the dye methylene blue (Sigma), because hybridization with probes specific for GAPDH gave inconsistent results. Probes were labelled with [α-32P]dCTP (3000 Ci/mmol) by random priming using the High Prime DNA labelling system (Roche). Specific activity was approximately 2 x 10⁹ dpm/µg of DNA. Membrane was prehybridized for 20 min at 60°C in QuikHyb solution (Stratagene). Hybridization was then performed in the presence of labelled probe at 55°C for 2 h in the same solution. Membrane was washed twice with 2 x SSC buffer (0.15 M NaCl/0.015 M Na₂-citrate, pH 7.6), 0.1% (w/v) SDS at RT for 15 min, followed by one wash with 0.1 x SSC, 0.1% SDS at 50°C for 30 min. Quantification was performed by scanning densitometry of the X-ray films (GS-800 Calibrated Densitometer, Bio-Rad).

RESULTS

Identification of differentially expressed mRNAs in 0.28AS-2 cells. The differential display technique was used to compare mRNA expression pattern of wild type EATC cells and 0.28AS-2 cells expressing antisense glutaminase mRNA. A total of six 5′-arbitrary primers were used. Each of the 5′-primers was paired with one of the six 3′-oligo(dT) primers (Table 1) and used to amplify cDNAs obtained by reverse transcription of total RNA from EATC and 0.28AS-2 cells. To prevent isolation of “false positives”, all amplification experiments were performed on two different dilutions of each cDNA sample. Differentially expressed bands were detected by comparison of 0.28AS-2 cells with EATC cells. Several representative DD autoradiographs using mRNA from both cell types are shown in Figure 1. Although there were cDNA bands that showed an increase in band intensity in 0.28AS-2 cells (e.g., bands 24 and 25), most of the potential positives detected were significantly down-regulated in the cells transfected with antisense glutaminase cDNA (e.g., bands 13, 22, 14 and 15). After scanning the autoradiographs of several DD gels, we selected a total of six differentially expressed cDNA bands (Table 2). These bands were extracted from the gels, reamplified and cloned.

Table 1 Primers used in differential display

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>ATTAACCCCTCAACTAATTGCTGGGA</td>
</tr>
<tr>
<td>P2</td>
<td>ATTAACCCCTCAACTAATTGCTGGAG</td>
</tr>
<tr>
<td>P3</td>
<td>ATTAACCCCTCAACTAAATGCTGTTG</td>
</tr>
<tr>
<td>P4</td>
<td>ATTAACCCCTCAACTAATGCGTGTAG</td>
</tr>
<tr>
<td>P5</td>
<td>ATTAACCCCTCAACTAAATGCTGGTG</td>
</tr>
<tr>
<td>P6</td>
<td>ATTAACCCCTCAACTAATGCTGTTG</td>
</tr>
<tr>
<td>T2</td>
<td>CATATGCTGAGTATCCTCTTTTTTACT</td>
</tr>
<tr>
<td>T3</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T4</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T5</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T6</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T7</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T8</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
<tr>
<td>T9</td>
<td>CATATGCTGAGTATCCTCTTTTTTAC</td>
</tr>
</tbody>
</table>

Northern blot analysis. Northern blot analysis was performed to confirm six selected cDNA bands. Total RNA was extracted from EATC and 0.28AS-2 cells. Specific cDNA probes for each putative positive were generated by PCR of the isolated band or by restriction analysis of commercially available...
Transcriptome Analysis of Tumor Cells Expressing Antisense Glutaminase mRNA

The clones 13 and 16 did not show significant differences in their expression levels in both cell types; therefore, they were considered as false positives. In glutaminase-inhibited 0.28AS-2 cells, the expression levels of clones 14, 15, 22, and 26 were significantly lower than in EATC cells; specifically, they showed increases in EATC cells ranging from 6-fold for clone 22 to 1.7-fold for clone 14 (Fig. 2).

Identification of cDNA clones. The nucleotide sequences of the differentially expressed cDNA clones were determined and compared with the NCBI and Ensembl databases using BLAST analysis (Table 2). Genomic and expressed sequence tag (EST) databases from mouse and human were used, because the cDNAs obtained in DD are 3'-based and the sequences are usually, but not always, at the untranslated region. The list of identified cDNAs after BLAST analysis is summarized in Table 2. Band 14 was identified as NEDD-4 ubiquitin-protein ligase, band 15 showed strong identity with FMNL3B or formin-like isoform 3-B, whereas bands 22 and 26 were assigned to mouse HMGA2 (high-mobility group, isoform 2) and USP-15 (ubiquitin carboxyl-terminal hydrolase 15) proteins, respectively.

DISCUSSION

Our research programme is committed to unraveling the molecular mechanisms underlying the reversion of the transformed phenotype achieved after specific antisense glutaminase inhibition in tumor cells. Using the DDRT-PCR technique, we have identified a number of genes whose expression is significantly altered in cells with blocked glutaminase expression. We first focused on those bands showing a clear altered expression pattern on the DD gels; only six bands were considered as relevant from the numerous bands obtained from 36 PCR reactions. After validation by Northern blot analysis, four bands were confirmed as real positives. We then performed sequencing of the selected clones and basic local alignment search tool (BLAST) searches with mouse and human data banks to identify the clones.

High-mobility group A2 (HMGA2) is a member of the Hmga gene family of nonhistone chromosomal proteins, often referred to as architectural transcription factors. It is known that transformed cells display many metabolic traits characteristic of the embryonic and fetal stages of development; hence, the downregulation of HMGA2 in 0.28AS-2 cells is fully consistent with that view. In addition, HMGA2 has been clearly implicated in cell proliferation and tumorigenesis: overexpression of HMGA2 has been reported in various malignant tumors, including breast cancer. Therefore, up-regulation of HMGA2 in EATC cells could be anticipated, keeping in mind that Ehrlich ascites tumor cells originally derived from a mouse mammary tumor. Formin-homology proteins, such as FMNL3B found in this study, belong to a family of multidomain scaffold proteins involved in actin-dependent morphogenetic events. The mouse formin-like 3 protein, isoform B, is down-regulated in 0.28AS-2 cells and contains the formin-homology (FH) domains FH1 and FH2, characteristics of many members of this family. The proline-rich FH1 domain mediates interactions with a variety of proteins, whereas the FH2 domain plays a crucial role in the reorganization of the actin cytoskeleton. The assignment of in vivo functions to formins is very complicated by the multiple isoforms
that a single cell may express and by the effect of different interactors. However, considering the dramatic change in morphology showed by 0.28AS-2 cells, compared with the parental EATC cell line, it is tempting to speculate that decreased FMNL3B expression could be ascribed to changes in actin nucleation and polymerization activities in the antisense cells, which may contribute to conform a more differentiated phenotype. In fact, 0.28AS-2 cells become polygonal and smaller in size, forming adherent clumps, in contrast with the fibroblast-like pattern of wild-type EATC cells. This change in cell morphology has to be based on cytoskeletal remodeling.

Two downregulated genes found in this work are related with the regulation of cell proteolysis. NEDD-4 is an ubiquitin-protein ligase participating in the ubiquitination of selective protein substrates, whereas ubiquitin-specific protease USP-15 is a deubiquitinating enzyme which catalyzes cleavage of ubiquitin (Ub) precursors, thus contributing to recycling of Ub. NEDD-4 was initially identified as a highly expressed transcript in the mouse embryonic brain, which subsequently decreased at the RNA level during neuronal differentiation.

Accordingly, human NEDD-4 was also down-regulated at the protein level by inducing differentiation of human SMS-KCN neuroblastoma. The authors conclude that these experimental evidences support the idea of NEDD-4 being involved in the cell growth and differentiation by allowing upregulation of yet unidentified target proteins. Therefore, downregulation of NEDD-4 in the more differentiated and slow-growing 0.28AS-2 cells seems quite consistent with those earlier results in brain tissue and tumor cell lines. On the other hand, it has been shown that neoplastic, particularly breast cancer cells, are characterized by an increased activity of the Ub/proteasome pathway, which could reflect a general response to hyperproliferation and stress.

The last gene identified in our analysis codes for USP-15 protein, the mouse ortholog of the human ubiquitin-specific protease USP-15. This protein is tightly bound to the proteasome and catalyzes the removal of Ub from ubiquitin-conjugated substrate proteins. Besides its main function in recycling Ub, attention has been given recently to other biological functions proposed for USP-15 and other deubiquitinating enzymes, including cell growth and differentiation, development, oncogenesis, neuronal diseases, chromosome structure and transcriptional regulation.

A highly-related murine USP-4 protein was first characterized as a proto-oncogene and, later on, the human and murine USP-4 proteins were demonstrated to interact with the retinoblastoma (Rb) family of tumor suppressor proteins, suggesting a possible mechanism for cellular transformation. Interestingly, human and mouse USP-15 also have the Rb interaction motif and share high sequence similarity with USP-4. Thus, one can speculate that USP-15 might collaborate in cell growth regulation by stabilization of Rb family members, since Rb is a target of the Ub/proteasome pathway.

There is growing evidence that Sp1 family of transcription factors plays an important role in proliferation and differentiation. Previous studies from our laboratory indicated that inhibition of glutaminase increases Sp1 and Sp3 expression. We also showed increased Sp1 transcriptional activity using a reporter plasmid containing six Sp1 consensus sites. This result does not imply that all Sp1-controlled genes are upregulated since Sp1 control of gene expression is gene-specific; that is, the response of Sp1-sites to a particular stimulus is promoter- and context-dependent. In fact, we have observed that MUC1, a Sp1 controlled protein, is down-regulated in cells expressing antisense glutaminase mRNA, and recent data indicate that Sp1 is often involved in the negative regulation of specific genes. The promoter region of Hmg2 has functional Sp1 sites and sequence analysis of the 5'-flanking region of Fmnl3, Nedd-4 and Usp-15 also reveals putative Sp1 consensus sequences. Therefore, Sp1 is a potential candidate for transcriptional regulation of these genes and would play a role in orchestrating the phenotypic changes underlying glutaminase inhibition.

In conclusion, we have demonstrated the validity of the differential display technique to delineate possible signaling and metabolic pathways underlying the reversion of a malignant tumor phenotype to a more differentiated, slow-growing phenotype, elicited after blocking glutaminase expression by antisense technology. Target genes identified in antisense-transformed cells will help us to unravel the molecular mechanisms involved in such response.

References
Transcriptome Analysis of Tumor Cells Expressing Antisense Glutaminase mRNA


