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Differentially Expressed cDNAs in PLCβ3-Induced Tumor Suppression in a Human Endocrine Pancreatic Tumor Cell Line: Activation of the Human Mismatch Repair Protein 3 Gene

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Phospholipase Cβ3 (PLCB3) is located to chromosome 11q13 in the vicinity of the multiple endocrine neoplasia type1 (MEN1) gene and shows loss of expression in some neuroendocrine tumors. Transfection of PLCB3 to neuroendocrine cell lines induces growth suppression and phenotypic alterations, but the mechanisms remain unclear. To investigate the underlying events behind this tumor suppression, we performed an RT-Differential cDNA Display of total RNA from BON-1 (human endocrine pancreatic tumor cell line) transfected with PLCB3 and compared to wild type and BON-1 transfected with vector without insert. PLCB3 transfection resulted in increased expression of 4 genes and decreased of 2. The two inhibited were homologous to S100A3 and Chromogranin A. One of the four activated cDNAs could be identified as human mismatch repair protein 3 mRNA (hMSH3), and another was homologous to TIS/MA-3 mRNA (mouse topoisomerase suppressor inhibited gene/mouse apoptosis gene-3). Differential expression of these genes may contribute to the PLCB3-induced tumor suppression of neuroendocrine tumor cell lines.

Key Words: **PLCB3 (phosphatidylinositol-specific phospholipase C β3 (human)); PLCAP (PLCB3 activated pathway); PLCIP (PLCB3 inhibited pathway); CgA (chromogranin A); hMSH3 (human mismatch repair protein 3); TIS/MA-3 (mouse topoisomerase suppressor inhibited gene/mouse apoptosis gene-3); QPCR (quantitative PCR).**

Phospholipase C β3 (PLCB3) frequently show loss of expression in sporadic endocrine tumors as well as MEN1 associated lesions and is located on chromosome 11q13 in the vicinity of the MEN1 gene (1, 2). We have recently described that transfection of PLCB3 to neuroendocrine tumor cell lines lacking endogenous expression induces suppression of the neoplastic phenotype (3). Little is known about the specific functions of PLCB3 but different members of the phospholipase C beta family are widely expressed in normal tissue, and are involved in the signal transduction of the seven transmembrane receptors. PLCs generate the second messenger molecules inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG upon binding activated subunits of heterotrimeric G proteins (4).

To identify downstream target genes in the PLCB3 signaling pathway that might be relevant, we used an RT-Differential cDNA Display strategy, using RNA from BON-1 wild type cells (human endocrine pancreatic cell line) and BON-1 stably transfected with PLCB3 (BON/PLC). We reasoned that genes differentially expressed between these two cell lines might contribute to the phenotypic changes induced by PLCB3 in neuroendocrine tumor cells. In this paper, we identify genes that are novel downstream targets to the PLCB3 signaling pathway. The expression of four genes are increased (PLCB3 activated pathway; PLCAP) and the expression 2 decrease (PLCB3 inhibited pathway; PLCIP) as a result of PLCB3 expression in BON-1 cells. Corroboration of the results was achieved either by using the obtained fragments, or the fullength cDNA as probes for Northern blotting or by using quantitative PCR.

MATERIALS AND METHODS

Cell cultures. We used a human endocrine pancreatic tumor cell line (BON-1) stably transfected with the coding region of *PLCB3* (1) (clone BON/PLC) as well as BON-1 wild type (BON/wt) and BON-1 transfected with the vector (pCEP-4, Invitrogene) without insert (BON/v). BON/wt has low protein expression of PLCβ3 compared to a panel of normal tissue and non-endocrine cell lines. Transfection of *PLCB3* to BON-1 cells induce growth inhibition both *in vitro* and *in vivo* and increases IP_3 levels (3). We used the episomal pCEP-4 vector to avoid unspecific genomic effects. Applied methods for cul-

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turing and transfection of BON-1 cells have been previously described (3).

Isolation of RNA and Northern blots. Total RNA was isolated and pooled in parallel with experiments showing growth inhibition (3), mRNA was isolated using PolyAtract (Promega, Madison, WI). Approximately 2 μg of mRNA from BON/wt, BON/v, and BON/PLC were used for Northern blot analysis as described (3). Northern blots were repeated two or three times. Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Relative expression was calculated comparing densitometric units between the signals from the BON/wt, BON/v and BON/PLC lanes. To control for variations in loading and transfer among samples the signals and subsequent calculations were normalized to β-actin signals obtained on the same blot.

RT-Differential cDNA display. Total RNA from BON/wt, BON/v, and BON/PLC was treated with DNase 1 to avoid contaminating genomic DNA. From 2 μg of total RNA (BON/wt, BON/v and BON/PLC), a first strand cDNA synthesis was performed according to the protocol included in the Delta Differential Display kit (Clontech, Palo Alto, CA). Differential display PCR was performed on the derived cDNAs using 10 arbitrary "P" primers and 9 oligo(dT) ("T") primers, giving 90 different pair wise combinations of "P" and "T" primers for each sample (primer sequences are available at the Clontech web site, www.clontech.com). Radioactive PCR was performed using [α-^{33}P]dATP (Amersham, Sweden) and Advantage KlenTaq Polymerase mix (with TaqStart Antibody) (Clontech) according to the protocol (Clontech). We used the Perkin-Elmer Thermal Cycler 480 for thermal cycling according to the protocol (Clontech). The PCR products from each primer combination for the three different cDNAs were denatured and loaded side by side on 0.4 mm thick denaturing 5% polyacrylamide/8 M urea (in 0.5× TBE buffer) gels. Gels were run on an electrophoresis apparatus (Life Technologies, Gaithersburg, FL) at 70 W for 2.75 h, then vacuum dried at 80°C for 1 h and exposed overnight using storage phosphor screens (Molecular Dynamics, Sunnyvale, CA). Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics). Differentially expressed bands were cut out using sterile scalpels. Gels were re-exposed to confirm that the right band had been cut. Bands were eluted in 40 μl of water for 5 min at 100°C, and then PCR reamplified with the original primers for 25 cycles as described above. PCR products were purified and used for direct sequencing. Sequence reactions was performed using dye terminator chemistry (DNA sequencing kit, ABI, Perkin-Elmer, Foster City, CA) and the original primers with thermal cycling on a GeneAmp PCR Systems 9600. Sequence gels were run and analyzed using the ABI 373A automated sequencer (Perkin-Elmer). Two of the differentially expressed genes generated readable sequences which showed high homology to known genes (S100A3 and Chromogranin A). The full-length cDNA for these genes were used as probes on Northern blots to confirm differential expression. PCR products that did not produce a readable sequence were assumed to contain contaminating bands. These products were cloned into the PCR II-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer protocol. Plasmid DNA was isolated from 10 positive clones from each cloning reaction using the Miniprep kit from Qiagen (Valencia, CA).

Plasmid DNA was sequenced as above using T7 and SP6 primers. The cloned PCR products generally contained 2 or 3 different sequences. The original primers were used for PCR to generate fragments from the different sequences. These were used to make probes for Northern blotting to verify differential expression. When there was no detectable signal on Northern analysis we assumed that this might be due to low abundant RNA (hMSH3 and PLCAP2). In these cases Quantitative PCR (see below) was used to verify the differential expression. All homology searches against GenBank was carried out using BLAST (NCBI). All sequences and primers used are available at request.

Quantitative PCR (QPCR). Relative RNA expression of hMSH3 and PLCAP2 in the BON/wt and BON/PLC cell lines was determined by real-time quantitative PCR (5). Gene-specific primers and fluorogenic probes (TaqMan probes) were designed and used to amplify and quantitate the hMSH3 and PLCAP2 transcripts from the same derived cDNAs as above. Relative quantity was measured against a standard curve generated from dilution series of target-specific PCR fragments of hMSH3 and PLCAP2 respectively (6). Primers and probes were designed using PrimerExpress software (ABI, Perkin-Elmer) and reactions were performed and analyzed using an Applied Biosystems PRISM 7700 Sequence Detector (Perkin-Elmer). At each set-up, three duplicates of each cDNA was used and repeated twice. The hMSH3 and PLCAP2 signals were normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. All TaqMan assay reagents were obtained from Perkin-Elmer Applied Biosystems.

RESULTS

Differential cDNA Display

In order to avoid false positives we isolated only "distinct" (band vs no band) differentially expressed bands from the experiments. This approach generated 8 bands of interest. The reamplified products from the selected bands were sequenced without subcloning and 4 of them generated readable sequence (PLCIP 1A, 1B, 2A, and 2B). PLCIP 1A and 1B were identical as well as PLCIP 2A and 2B. Homology search (BLAST, NCBI) revealed >95% homology with S100A3 (GenBank Accession No. NM002960) for PLCIP 1A and 1B and with Chromogranin A (Accession No. NM001275) (CgA) for PLCIP 2A and 2B. We found it necessary to subclone the remaining re-amplified products to obtain single recombinant clones. Sequencing was performed on 10 recombinant subclones for each selected band. Each of the subcloned bands proved to contain 2–3 different clones.

Differential Expression

Using probes for S100A3 and CgA made from full-length cDNA on Northern blots, we found that both of these transcripts were down-regulated in the BON/PLC cell line compared to the parent BON/wt cells and BON/v, showing a relative expression of 40 and 35% respectively (Fig. 1). Using probes made from the single recombinant clones we identified 2 up-regulated bands in the BON/PLC cell line. PLCAP 3 and 4 showed increased expression in BON/PLC by 162 and 65% respectively compared to controls, but did not show any homology to known genes (Fig. 2). However, PLCAP3 had 100% homology to a draft sequence on chromosome 3q (clone RP11-484D18, Accession No. AC023235), and PLCAP4 matched a draft sequence on chromosome 5q to a 100% (clone CTB-77M18, Accession No. AC010313). We assume that the remaining two differentially expressed bands (PLCAP1 and 2) corresponded to low abundant RNA transcripts not readily identified by Northern analysis. To verify their differential expression we instead used quantitative

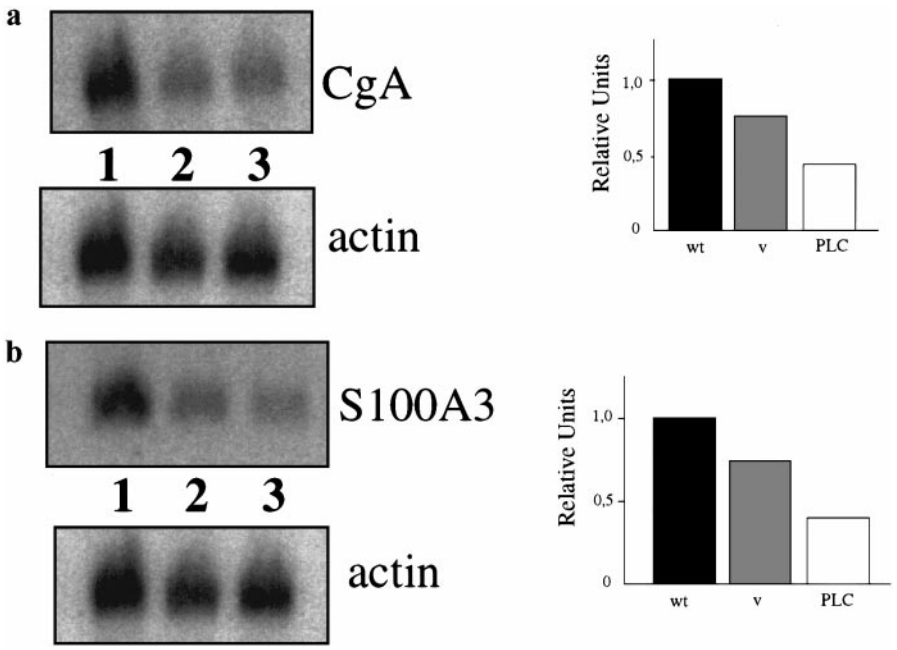

FIG. 1. Differential expression verified by Northern blot. (a) Down-regulation of Chromogranin A (CgA) mRNA expression in the BON-1 cells transfected with PLCB3 (BON/PLC) compared to the controls: BON-1 wild type (BON/wt) and BON-1 transfected with empty vector (BON/v). (b) Down-regulation of S100A3 mRNA expression in the BON/PLC cell line. Calculated values of relative densitometric units are normalized to β-actin obtained on the same blot. Lane 1, BON/wt; Lane 2, BON/v; Lane 3, BON/PLC.

PCR (QPCR). PLCAP 1 (200 bp) shared 99% homology with the mRNA for the human mismatch repair gene 3 (hMSH3, Accession No. NM002439) between bases 2850 and 3037. By using QPCR we were able to identify PLCAP 1 as up-regulated by 26 and 13 times in the BON/PLC cell line compared to BON/wt and BON/v respectively. The PLCAP2 transcript (170 bp) does not match any known human transcript but shares 99% homology to a contig on chromosome 10q (clone RP11-313D6, Accession No. AL136368). PLCAP2, however, matched the mRNAs TIS (mouse topoisomerase suppressor inhibited gene) and MA-3 (mouse apoptosis gene-3). TIS (2196 bp, Accession No. D86344) and MA-3 (2181 bp, Accession No. D50465) are two independently cloned mouse cDNAs that share 99% homology, with a consensus sequence of 2180 bp (7, 8). PLCAP2 matched the consensus sequence to 90% between bases 1818 and 1957 and was up-regulated in the BON/PLC cell line by 6 times compared to both BON/wt and BON/v (Table 1). A summary of primers and homologies are presented in Table 2.

DISCUSSION

We have recently shown that restoration of the PLCB3 expression in neuroendocrine tumor cell lines lacking endogenous expression, will suppress cell growth and alter the phenotype indicating that PLCB3 might be important in neuroendocrine tumorigenesis (3). In the present paper we have used RT-Differential display to be able to isolate 6 different cDNAs that are differentially expressed as a result of PLCB3 being transfected to neuroendocrine cells (BON-1). We verified the differential expression using Northern blots and QPCR. CgA turned out to represent one of the two mRNAs that were inhibited by PLCB3 expression. CgA is co-secreted along with hormones in neuroendocrine cells and is used as a clinical marker for neuroendocrine tumors. It has also been suggested that CgA might be an autocrine growth factor for neuroendocrine cells (9). S100A3 was also found to be down-regulated in the PLCB3 transfected BON-1 cells. The S100 protein family belongs to the superfamily of calcium-binding proteins of the EF-hand type (10, 11). There is no information regarding function or physiologic properties of the S100A3 protein available. One study reports that the S100A3 mRNA expression is inversely correlated to breast cancer progression (12), and one study show that the expression of S100A3 protein is increased in anaplastic astrocytomas compared to low-grade astrocytomas (13). S100A4, however, is known to induce metastatic phenotype in otherwise non-metastatic tumor cells, correlate to metastatic disease and worse prognosis in several tumor forms and seem to be involved in cell motility, as well as specific phosphorylation events in the cell, acting through PKCs (14). Another S100 member, S100B has been implicated to be involved in the regulation of Ndr, which is involved in the regulation of cell division (15). We hypothesize that PLCB3 might down-regulate

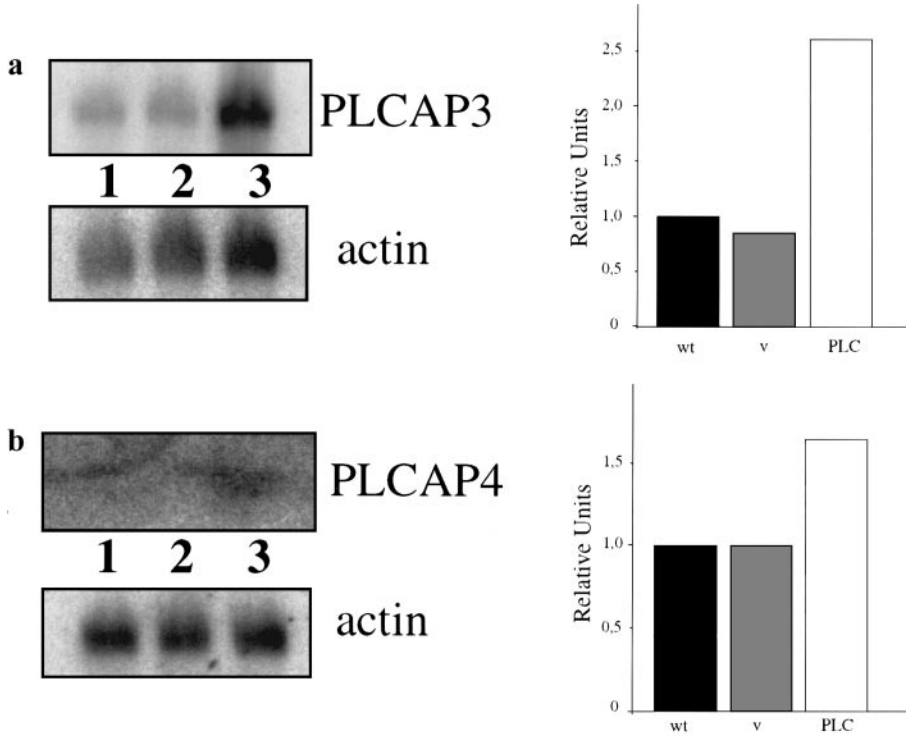

FIG. 2. Differential expression verified by Northern blot. (a) Up-regulation of PLCAP3 mRNA expression in the BON-1 cells transfected with PLCB3 (BON/PLC) compared to the controls: BON-1 wild type (BON/wt) and BON-1 transfected with empty vector (BON/v). (b) Up-regulation of PLCAP4 mRNA expression in the BON/PLC cell line. Calculated values of relative densitometric units are normalized to β-actin obtained on the same blot. Lane 1, BON/wt; Lane 2, BON/v; Lane 3, BON/PLC.

S100A3 through cross-talk with other pathways involved in regulating the expression of S100 proteins. It has been indicated that silencing of the EGF receptor (EGFR) activity, when no ligand is present, requires the presence of PLCB (16). There are several reports of crosstalk between PLC mediated signal transduction pathways and for instance the retinoic acid and adenylyl cyclase pathways (17, 18). S100A2 is, at least in part, regulated through an EGFR dependent mechanism (19). It is known that β-cells respond to betacellulin, a member of the EGF family, with mitogenic signals (20). One might speculate if the lack of PLCB3 activity would lead to continuous EGFR activity with growth promoting effects and if the transcription of S100A3 is controlled through this mechanism.

TABLE 1

Relative Expression of hMSH3 and TIS/MA-3 Using Real-Time Quantitative PCR

cDNA clone	BON/wt	BON/v	BON/PLC
hMSH3	13 ± 4	26 ± 2	350 ± 48
TIS/MA-3	40 ± 11	43 ± 12	224 ± 23

Note. Expression levels in cDNA derived from BON1 wild type (BON/wt), BON-1 transfected with vector only (BON/v), and BON-1 transfected with PLCB3 (BON/PLC).

The expression level of a member of the human mismatch repair system, hMSH3, was found to be up-regulated in the PLCB3 transfected cells. It is unclear how the expression of the different members of the mismatch repair system is regulated. It has been reported that silencing of hMSH3 is involved in hematologic malignancies as well as in Heriditary Non-Polyposis Colon cancer (21, 22). Not only silencing, but

TABLE 2

Characteristics of Differentially Expressed cDNA Clones

Differentially expressed clone	Primers	mRNA, kb	Homology
PLCIP1A	P8/T7	0,7	S100A3[a]
PLCIP1B	P8/T8	0,7	S100A3
PLCIP2A	P8/T3	1,8	CgA[b]
PLCIP2B	P9/T4	1,8	CgA
PLCAP1	P5/T6	3,5	hMSH3[c]
PLCAP2	P5/T6	unknown	mouse TIS/MA-3[d]
PLCAP3	P8/T3	2	draft seq. chr 3q
PLCAP4	P1/T7	4	draft seq. chr 5q

[a] Human calcium binding protein S100A3 mRNA.
[b] Human chromogranin A mRNA.
[c] Human mismatch repair protein 3 mRNA.
[d] Mouse topoisomerase inhibitor suppressed gene mRNA/mouse apoptosis 3 gene mRNA.

an imbalanced expression of hMSH3 and hMSH6 might lead to mismatch repair deficiency (23). There are also reports showing that transfection of a mismatch repair gene to cells lacking endogenous expression lead to suppression of growth (24). The intranuclear localization of PLCB3 (25, 26) and the studies of PLCB signaling in the nucleus related to DNA synthesis and proliferation (27–29) suggest that PLCB3 signaling in the nucleus might be involved in the regulation of hMSH3 transcription. One might speculate if PLCB3, indirectly by increasing the expression of hMSH3, might contribute to a stabilization of the genome.

PLCAP2 had a match of 90% to the mouse mRNAs for TIS and MA-3, which share a consensus sequence of 99% spanning almost the entire transcript. Although no known human transcript match this cDNA clone it is intriguing to speculate if this is a part of the human homologue to any of these transcripts possibly located on human chromosome 10q. TIS is found to be down-regulated when treating RVC lymphoma cells with topoisomerase inhibitors and MA-3 is up-regulated in programmed cell death in mouse (7, 8).

In summary, the amplification of four genes (hMSH3, TIS/MA-3, and 2 unknown) and the repression of two (S100A3 and CgA) in BON-1 cells transfected with PLCB3 may indicate an important role for these genes in neuroendocrine tumorigenesis, and provide novel insights to the role of PLCB3 in regulating gene expression.

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