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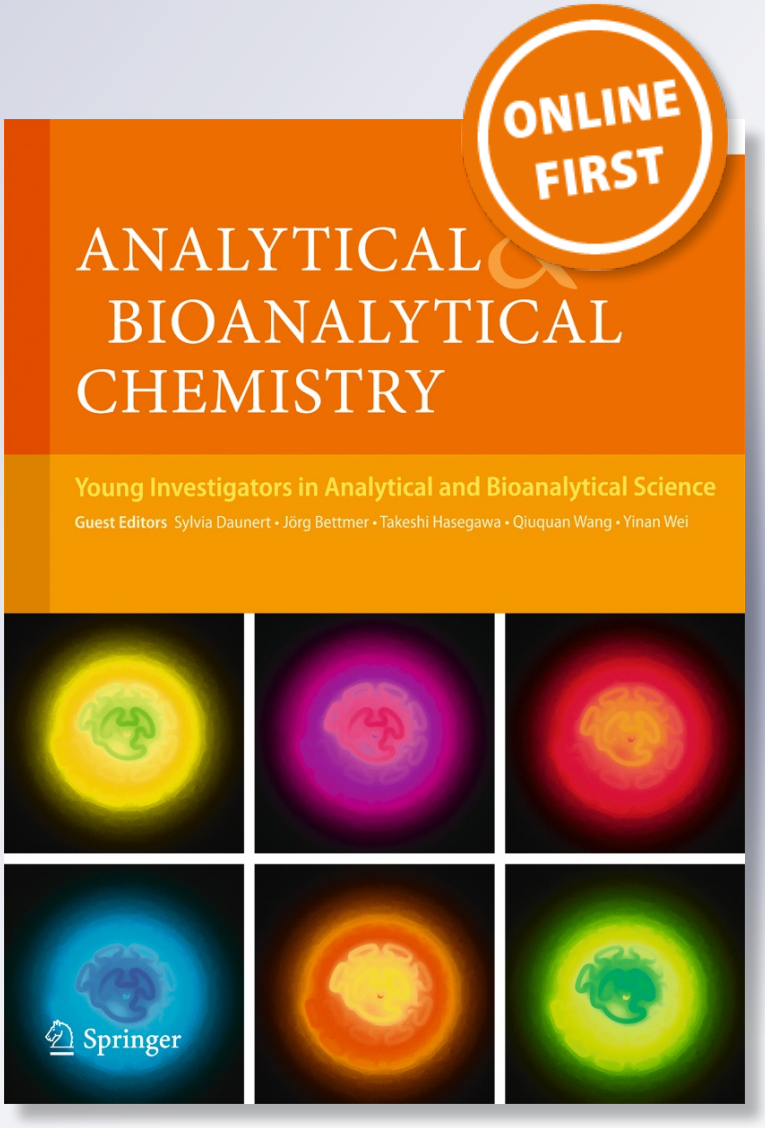
Analytical and Bioanalytical Chemistry

ISSN 1618-2642

Anal Bioanal Chem
DOI 10.1007/s00216-012-6182-5



Springer

Anal Bioanal Chem
DOI 10.1007/s00216-012-6182-5

ORIGINAL PAPER

Effect of transfection with *PLP2* antisense oligonucleotides on gene expression of cadmium-treated MDA-MB231 breast cancer cells

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Received: 13 April 2012 / Revised: 23 May 2012 / Accepted: 4 June 2012

Abstract Emerging evidence indicates that cadmium (Cd) is able to regulate gene expression, drastically affecting the pattern of transcriptional activity in human normal and pathological cells. We have already shown that exposure of MDA-MB231 breast cancer cells to 5 μM $CdCl_2$ for 96 h, apart from significantly affecting mitochondrial metabolism, induces modifications of the expression level of genes coding for members of stress response-, mitochondrial respiration-, MAP kinase-, NF–κB-, and apoptosis-related pathways. In the present study, we have expanded the knowledge on the biological effects of Cd–breast cancer cell interactions, indicating *PLP2 (proteolipid protein-2)* as a novel member of the list of Cd-upregulated genes by MDA-MB231 cancer cells and, through the application of transfection techniques with specific antisense oligonucleotides, we have demonstrated that such over-expression may be an upstream event to some of the changes of gene expression levels already observed in Cd-treated cells, thus unveiling new possible molecular relationship between PLP2 and genes linked to the stress and apoptotic responses.

Keywords Cadmium · PLP2 · Breast cancer · Differential display-PCR · Caspase · Gene expression

Published in the topical collection *Metallomics* with guest editors Uwe Karst and Michael Sperling.

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Introduction

The industrial and environmental pollutant cadmium (Cd) is known to intervene in biological systems by accumulating into living cells via import through different cell surface systems, e.g., ZIP transporters, Fe^{++}/H^{+} cotransporter divalent metal transporter 1, and CaV3.1 and −3.2 T-type Ca^{++} channels and targeting the Zn-binding domains of several metalloproteins, thereby interfering with or preventing Zn-dependent cellular functions [1, 2]. Cd is not essential for the human body and has no known useful biological functions. On the other hand, its effective role in both cancer initiation/progression, mostly due to its activating action on oncogenes via β-catenin and in apoptotic promotion of normal and tumoral cytotypes has been widely recognized [3, 4]. Noteworthy is the increasing evidence which indicates that Cd is capable of regulating gene expression, thereby acting as a transcriptional modulator for both prokaryotic and lower/upper eukaryotic cells, in normal and pathologic conditions [5].

We have previously reported that, when the highly tumorigenic triple-negative MDA-MB231 cell line, selected as an in vitro model of "aggressive" breast carcinoma, is treated for 96 h with $CdCl_2$, such exposure determines a decrease of cell proliferation with a 50% inhibitory concentration (IC_{50}) of 5 μM, a concentration similar to levels encountered in occupational exposure. We also showed the effect of metal treatment on several biological functions, such as the significant increase of mitochondrial respiratory activity, the impairment of intramitochondrial protein import process, and the accumulation of reactive oxygen species. Interestingly, additional evidence demonstrated the effect of 5 μM $CdCl_2$ exposure for 96 h on

Published online: 23 June 2012

the modification of the expression level of selected genes, including those coding for members of stress response-, mitochondrial respiration-, MAP kinase-, NF–κB-, and apoptosis-related pathways, by MDA-MB231 cells, which therefore appeared to be a useful cell model system for the study of the transcriptional modulation exerted by the metal [6–9].

In order to expand the list of Cd-responsive genes involved in the cytotoxic action exerted by the metal on the breast cancer cell line under study, here we have focused our attention on a novel differentially displayed cDNA product arising from polymer chain reaction (PCR)-mediated analysis of transcript arrays, i.e., that corresponds to a portion of *proteolipid protein 2* (*PLP2*) coding sequence. In the present paper, we report that exposure to $CdCl_2$ promotes the upregulation of *PLP2* expression by MDA-MB231 cancer cells and, through the application of transfection techniques with specific antisense oligonucleotides (asODN), we demonstrate that such over-expression may be an upstream event to changes of gene expression levels in Cd-treated cells, the already-observed upregulation of *caspase 8* being noteworthy [6], thus unveiling the new possible molecular relationship between PLP2 and genes linked to the stress and apoptotic responses. We also propose that *PLP2* could be taken into consideration as an additional putative gene expression signature to serve in both prognostic and predictive assessments for the health risk of exposure to environmental Cd.

Materials and methods

Cell cultures and transfection

The MDA-MB231 breast cancer cell line was routinely grown in RPMI 1640 medium plus 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/L amphotericin B (Invitrogen, Carlsbad/CA, USA), at 37 °C in a 5% CO_2 atmosphere. The HB2 breast epithelial cell line (courtesy of Cancer Research, UK) was routinely grown in high glucose–DMEM medium plus 10% fetal calf serum (Invitrogen), 5 μg hydrocortisone/ml (Sigma, St. Louis, MO/USA), 10 μg bovine insulin (Sigma), and the same antibiotic/antimycotic mixture, at 37 °C in a 5% CO_2 atmosphere. For Cd treatment, cells were plated in the presence of 5 μM $CdCl_2$ and grown for 96 h.

A directed antisense strategy was applied to downregulate *PLP2* expression, as already described [10]. The sequence of the phosphorothioate asODN targeted to PLP2 was 5′-GTG GTTTCGAGGACGCTGT-3′, complementary to bases 8–27 of its mRNA. The RNAdraw software [11] was utilized to predict the secondary structure of PLP2 mRNA (Fig. 1). The asODN was delivered to cells as a complex with Lipofectin (Invitrogen), according to manufacturer's instructions, in serum- and antibiotic/antimycotic-free medium. Control assays were performed with Lipofectin only.

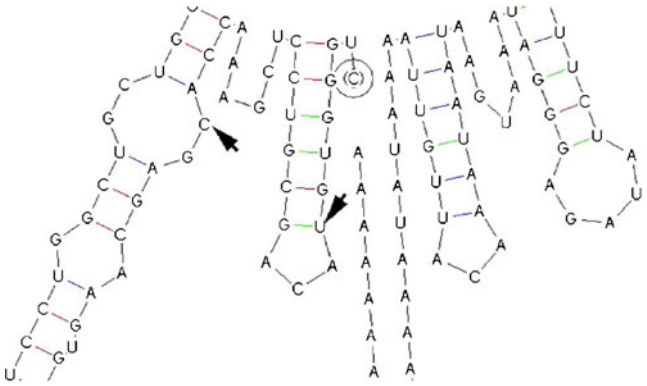


Fig. 1 Detail of the secondary structure of the human PLP2 mRNA as predicted by the RNAdraw program. The *arrows* point to the extremities of the sequence recognized by *PLP2* asODN

Crystal violet assay

Aliquots of 10^4 cells were plated into 96-well plates and grown for 96 h in the presence of either $CdCl_2$/Lipofectin or $CdCl_2$/Lipofectin/PLP2 asODN. For indirect cell number evaluation, nuclei were stained with 0.2% crystal violet in 2% ethanol, the dye eluted with 1% sodium dodecylsulfate (SDS) and quantitated spectrophotometrically at λ=570 nm.

RNA extraction and reverse transcription

Isolation of total RNA was carried out with Trizol reagent (Sigma). Before the reverse transcription, the total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and its quality and integrity checked through agarose gel electrophoresis in denaturing conditions. Enrichment of samples for mRNAs, as required for differential display-polymerase chain reaction (DD-PCR), was achieved by treatment with Terminator™ 5′-Phosphate-Dependent Exonuclease (Epicentre, Madison, WI/USA), following manufacturer's instructions. The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) in the presence of 100 ng random 6-mer primers (Sigma), 50 U RNase inhibitor (Promega), and 0.5 mM each of dNTPs; reverse transcription was carried out for 60 min at 42 °C, followed by treatment with 2 U RNase H (USB, Cleveland, OH, USA) for 20 min at 37 °C. The quality of cDNA was checked by amplification of "housekeeping" 18S cDNA

Differential display-polymerase chain reaction (DD-PCR)

For differential expression analysis, DD-PCR experiments were performed as already reported [e.g., 9, 12] using the arbitrary 10-mer primers designed by Sokolov and Prockop [13], in combinations of two. The PCR amplification was carried out using 25 pmoles of each of two primers, 1–2 μl

of the cDNA template, and 3.6 U of AmpliTaq DNA Polymerase, Stoffel fragment (Perkin Elmer, USA), in 50 μl of the appropriate reaction mixture. The thermal cycle used was a denaturation step of 94.5 °C for 3 min, followed by 45 cycles of 94.5 °C for 1 min, 34 °C for 1 min, 72 °C for 1 min, and a final extension of the product for 10 min at 72 °C.

After PCR amplification, 8 μl of the amplification products were electrophoresed in a non-denaturing 6% polyacrylamide gel in a sequencing apparatus at constant 55 W and the band pattern visualized via silver staining. Evaluation of band size was performed with SigmaGel software (SPSS, USA). For re-amplification of the selected differentially displayed band, the silver-stained gel was exhaustively washed with double-distilled water and the band of interest carefully scratched from the gel with a sterile syringe needle and used as template for PCR amplifications performed as described in the following section. Several cycles of amplification and electrophoresis were repeated until a single pure band was visualized in the gel and eluted using Ultrafree DA filter columns (Millipore, Bedford MA/USA).

The sequence of the purified PCR product was subsequently determined by MWG Biotech sequencing service, and DNA sequence similarity was searched with the BLAST algorithm [14] available online.

Conventional PCR

PCR analysis was performed using 2.5 μM of appropriate sense and antisense primers, 1 U RedTaq DNA polymerase (Sigma)/μl, 200 μM each of deoxyribonucleotides diphosphates, and 1 μl of the cDNA template obtained from total RNA. The latter one was omitted in negative controls. All the primers used were from Luparello et al. and Luparello and Sirchia [10, 15, 16]. The thermal cycle used was a denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, the appropriate annealing temperature for 30 s, and 72 °C for 30 s. A final extension of the product was performed for 5 min at 72 °C. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by Gel Red staining (Biotium, Hayward, CA, USA) under UV light

Semi-quantitative "multiplex"

Semi-quantitative "multiplex" (SM-PCR) was carried out as previously reported [e.g., 12]. Essentially, the cDNA species of interest was co-amplified with cDNA from 18S rRNA over a range of cycles, followed by 2% agarose electrophoresis and Gel Red stain. Cycle profile was a denaturation step of 94.5 °C for 3 min., followed by cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension of the product for 5 min at 72 °C. Cycles were limited to the minimum necessary for detection. The intensities of the bands of interest, evaluated with SigmaScan software, were normalized for those of 18S, and plotted as a function of cycle number. Exponential regression equations fitted to the curves were used to calculate the number of cycles necessary to reach a normalized intensity threshold value=1 for each sample. The relative difference in abundance between two samples was taken as 2^n where n is the difference between the numbers of cycles required by the samples to reach the threshold. Two different RNA preparations from each experimental condition were pooled to make the differences more significant between the expression levels, if any.

Statistics

The results in histograms are presented as average±SEM of triplicate experiments; the SEM is indicated as vertical bars in the figures. Data were analyzed using software-assisted analysis of variance (SigmaStat v.2; SPSS, Chicago, IL, USA), and $p<0.05$ was taken as the minimal level of statistical significance.

Results

PLP2 is upregulated in Cd-treated MDA-MB231 breast cancer cells

In order to search for novel genes whose modification of expression level might be linked to the lethal phenotype induced by Cd exposition, in a first set of assays, we submitted cDNA preparations, obtained from MDA-MB231 cells grown in the presence of 5 μM $CdCl_2$ for 96 h, to DD-PCR as described. Among the several differentially displayed bands present in the gel, we focused our attention on an amplification band of about 110 bp, obtained utilizing the BS76/BS78 combination of arbitrary primers, which was present only in the electrophoretic lane corresponding to the cDNA preparation from metal-treated cells (Fig. 2a). The differentially displayed cDNA fragment was purified as described and submitted to sequencing. Using BLASTN software, homology (score=111 bits, expect=3e–22) was found in the non-redundant nucleic acid sequence database (nr–nt) between the sequence obtained and that of region 6158–6217 of the coding sequence for *Homo sapiens proteolipid protein 2 (PLP2)* (Acc. nr. NG_016420), a.k.a. *A4 differentiation-dependent protein* (*A4*) [17].

We then checked the differential expression of *PLP2* by conventional PCR amplification in the presence of specific primers. As shown in Fig. 2b, after the PCR amplification, we found a product of the expected size (150 bp) in both experimental conditions, indicating that expression of *PLP2* was switched-on in both control and Cd-exposed cells. For semi-quantitative evaluation, the cDNA preparations from

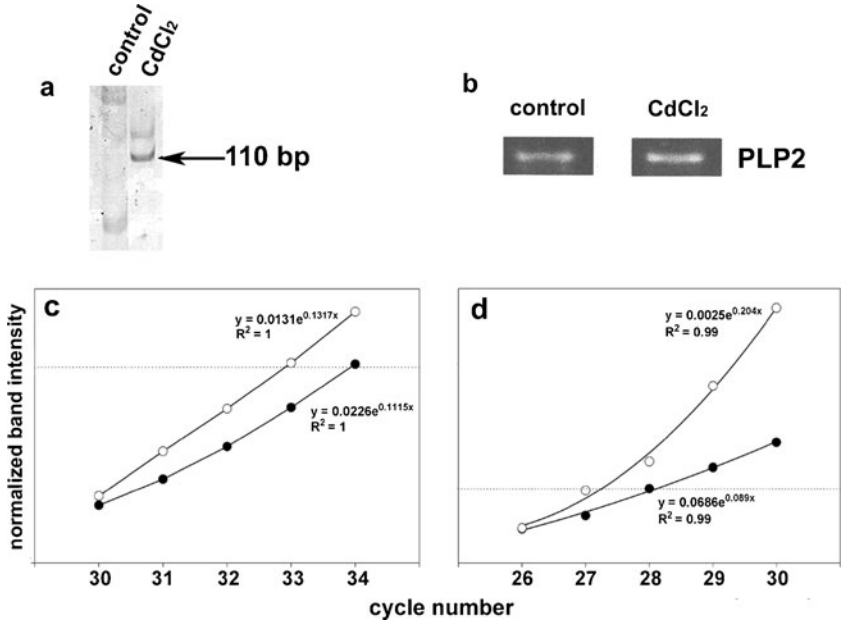


Fig. 2 Identification of *PLP2* as a differentially expressed gene in Cd-treated MDA-MB231 and HB2 cells. **a** DD-PCR of cDNA preparations from control and Cd-treated MDA-MB231 cells. The *arrow* points to a band of about 110 bp, selectively displayed in the sample obtained from cells exposed to the metal. Detail of a 6% sequencing polyacrylamide gel, *silver* staining. **b** Conventional PCR amplification of the product of *PLP2* cDNA in control and Cd-treated MDA-MB231 cells. Cycle number was 35. Agarose gel 2%, Gel Red stain. **c**, **d** SM-PCR for *PLP2*. Representative plot of normalized data versus cycle numbers fit with an exponential curve for MDA-MB231 (**c**) and HB2 (**d**) cells grown in control conditions (*black circle*) and in the presence of $CdCl_2$ (*white circle*). The *dotted line* represents the value=1

cells cultured in control conditions and in the presence of $CdCl_2$ were submitted to SM-PCR, as described. Figure 2c shows a plot of normalized data for *PLP2* versus cycle number, with exponential curve fits. Calculation of the relative difference in transcript abundance revealed that treatment of MDA-MB231 cells with 5 μM $CdCl_2$ for 96 h promoted upregulation of *PLP2* by about twofold with respect to control cells, thus validating the DD-PCR findings.

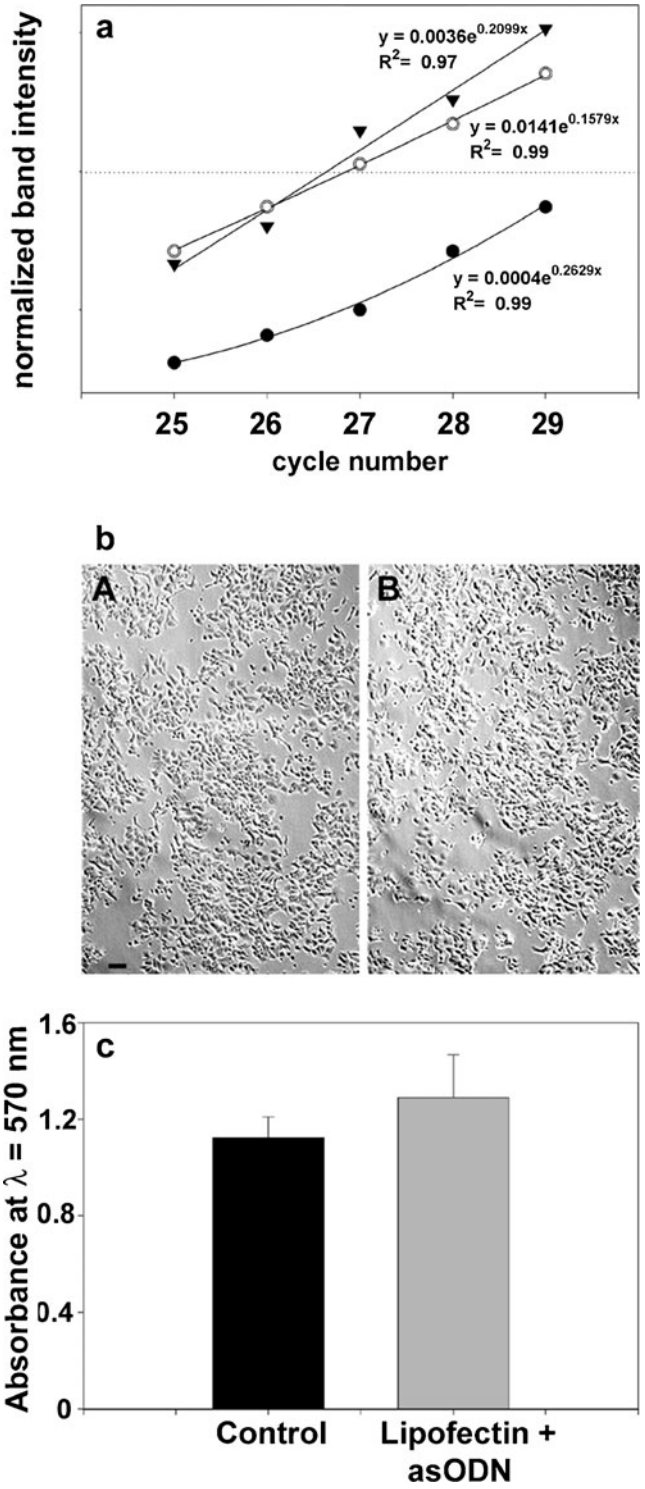
In a parallel assay, non-tumoral HB2 epithelial breast cells were submitted to the same exposure to the metal and the cDNA preparations from control and treated samples analyzed by SM-PCR for *PLP2*, once an amplification band of the expected size in both experimental conditions (not shown) was found. As shown in Fig. 2d, similar to what was found for MDA-MB231 cancer cells; also in HB2 cells, $CdCl_2$ treatment promoted upregulation of *PLP2* by about twofold, thereby suggesting a possible generalized effect of the metal on the modulation of such transcriptional activity.

In order to study the kinetics of Cd-dependent modulation of *PLP2* gene expression, preparations of control and treated MDA-MB231 and HB2 cells obtained after shorter-term exposures to 5 μM $CdCl_2$ (24 and 48 h) were submitted to SM-PCR. Since no significant difference was found for *PLP2* expression levels between control and treated cells of both lines at these time points (data not shown), we can assume that *PLP2* upregulation is a late event triggered by $CdCl_2$ treatments.

Antisense-mediated downregulation of PLP2 in Cd-treated MDA-MB231 cells is unable to affect cell morphological appearance and proliferation rate

In order to investigate whether the increase of *PLP2* expression levels might in some way be implicated in the already-reported Cd-triggered modifications of cell morphology, growth rate, and expression levels of stress response- and apoptosis-related genes [6, 9]; in a second set of assays, we induced asODN-mediated downregulation of PLP2 in MDA-MB231 cells exposed for 96 h to 5 μM $CdCl_2$ and evaluated both the appearance and density of the cell culture under the phase contrast microscope and cell number via crystal violet assays in parallel with vector only-treated cells and non-transfected cells, both grown in the presence of 5 μM $CdCl_2$ for 96 h, used as controls.

Preliminarily, we checked at mRNA level whether exposure to the specific asODN was successful in downregulating *PLP2*, through PCR amplification of cDNA preparations. As shown in Fig. 3a, incubation with Lipofectin-vehiculated PLP2-asODN resulted in the prominent downregulation (of approximately sevenfold) of *PLP2*, thereby confirming the success of the transfection technique and asODN chosen that



◀ **Fig. 3** Effect of transfection with *PLP2* asODN on *PLP2* expression, morphological appearance, and number of Cd-treated MDA-MB231 cells. **a** SM-PCR for *PLP2*. Representative plot of normalized data versus cycle numbers fit with an exponential curve for Cd-treated MDA-MB231 cells either non-transfected (*black down-pointing triangle*), transfected with Lipofectin only (*white circle*) or with Lipofectin+*PLP2* asODN (*black circle*). The *dotted line* represents the value=1. **b** Representative phase contrast micrographs of Cd-treated MDA-MB231 cells either non-transfected (*A*) or transfected with *PLP2* asODN (*B*). *Bar*=20 μm. **c** *Histogram* showing the absorbance of crystal violet eluted after lysis of stained MDA-MB231 cells cultured in the same experimental conditions as above. The *vertical bars* indicate the SEM of triplicate experiments; p was >0.05

was able to decrease gene expression down to levels even lower than that observed in unexposed MDA-MB231 cells. In addition, we also checked and confirmed that incubation with Lipofectin only did not modify the appearance and number of Cd-exposed MDA-MB231 cells (data not shown).

The results obtained in this set of experiments indicate that downregulation of *PLP2* exerts no effect on cell morphology and growth rate of MDA-MB231 cells exposed to the metal. Figure 3b shows that, at the microscopic observation, both untreated and asODN-treated cells displayed similar appearance and density onto the culture plate. In line with the observations made, the absorbance value of eluted crystal violet of PLP2 asODN-treated cells was comparable to that of untreated cells grown in $CdCl_2$-containing media (Fig. 3c), thereby suggesting that Cd-dependent *PLP2* upregulation was not directly responsible of the drastic changes in the adhesive and survival/proliferative behavior of the cell population.

Antisense-mediated downregulation of PLP2 in Cd-treated MDA-MB231 cells affects the expression levels of selected heat-shock and caspase genes

We then examined whether the downregulation of *PLP2* experimentally induced in Cd-treated MDA-MB231 tumor cells might result in changes of the expression levels of those stress response-related genes (*hsf1*, *hsp27*, *hsp-75*, *hsp-90β*, *hsc70*, and *grp78*), apoptosis factor genes (*Bcl-2*, *Dap kinase*, *Waf-1*), and caspase genes (*caspase-1*, *caspase-2*, *caspase-6*, *caspase-7*, *caspase-8*, and *caspase-9*), whose Cd-dependence was reported in a previous publication [6].

First, we submitted cDNA preparations obtained from untreated, vector only- and PLP2 asODN-treated cells exposed to $CdCl_2$ to conventional PCR to check whether the selected genes were expressed. As shown in Fig. 4a, amplification bands for all the tested cDNAs could be observed in all sets of samples, confirming that the specific gene expression was switched-on in control, vector only- and asODN-transfected cells.

To check whether gene expression levels were differing among the three experimental conditions, we then submitted the cDNA preparations to SM-PCR. As shown in the panel of graphs in Fig. 4b, no difference was found between the two controls, i.e., untreated and vector only-treated cells, whereas, when compared with controls, transfection with PLP2 asODN resulted in the upregulation of *caspase-1* (approx. tenfold), *caspase-2* (approx. fivefold), *caspase-9* (approx. fivefold), and *hsp27* (approx. twofold), and in the downregulation of *caspase-8* (approx. 4.2-fold) and *hsc70* (approx. 4.4-fold).

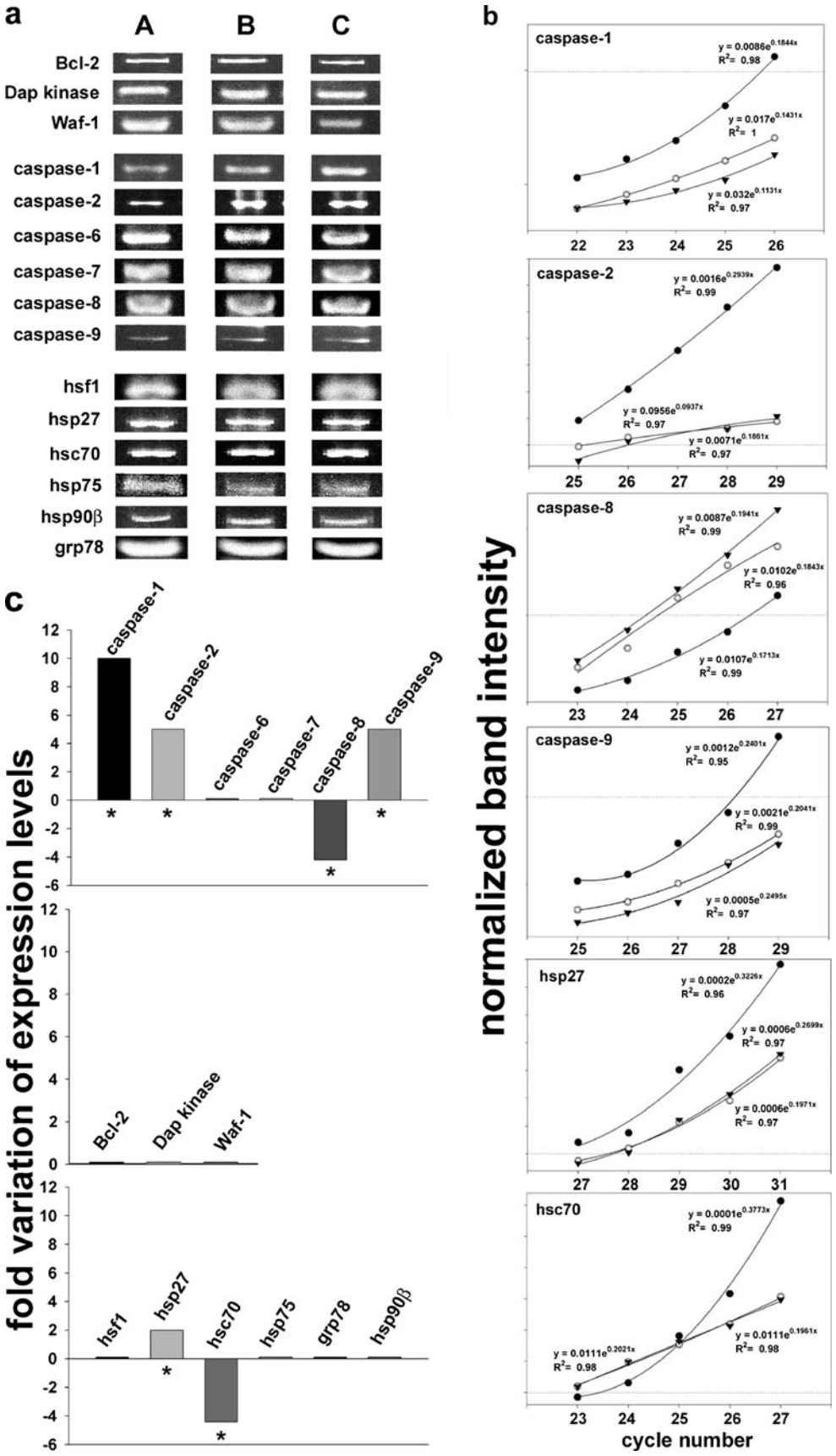


Fig. 4 Effect of transfection with *PLP2* asODN on stress response and apoptosis-related gene expressions by Cd-treated MDA-MB231 cells. **a** Panel showing the bands obtained from PCR amplification of cDNA preparations from Cd-treated MDA-MB231 cells either non-transfected (*A*), transfected with Lipofectin only (*B*) or with Lipofectin+*PLP2* asODN (*C*). PCR cycle number was 35. **b** SM-PCR for *caspase-1*, *caspase-2*, *caspase-8*, *caspase-9*, *hsp27*, and *hsc70.* Representative plots of normalized data versus cycle numbers fit with an exponential curve for Cd-treated cells either non-transfected (*black down-pointing triangle*), transfected with Lipofectin only (*white circle*) or with Lipofectin+*PLP2* asODN (*black circle*). **c** Histogram showing the average changes in expression levels of the tested genes based on SM-PCR assays of Cd-treated MDA-MB231 cells transfected with *PLP2* asODN compared with non-transfected controls (SEM of triplicate assays was less than 5% in each experimental condition). *=$p<0.05$

This suggests that *PLP2* expression may be upstream and in some way involved in the balance of hsp- and caspase mRNA synthesis, which in turn is implicated in cell protection and survival/death decisions. No significant difference was found for the expression levels of *hsf1*, *hsp75*, *hsp90β*, *grp78*, *Dap kinase*, *Bcl-2*, *Waf-1*, *caspase-6*, and *caspase-7*. The histograms in Fig. 4c report a synopsis of the expression level ratios of the studied genes in PLP2 asODN-transfected cells versus control cells, both cultured for 96 h in the presence of 5 μM $CdCl_2$.

Discussion

It is generally acknowledged that information at a molecular level is a necessary complement to descriptive epidemiological investigations on the effect of exposure of humans to pollutants, providing a list of marker genes useful for risk assessment. In the last decade, several molecular biomarkers have been validated as specific and reliable for Cd-induced toxicity, such as genes which encode for metallothioneins, for factors involved in the response to oxidative stress and in the onset of apoptosis, for signal transduction-linked receptors, and for miRNAs [5].

In the present work, we have reported the differential expression of *PLP2* by neoplastic MDA-MB231 cells as a response to culture in the presence of $CdCl_2$ at a concentration corresponding to the IC_{50} after long-term exposure and the effect of *PLP2* downregulation on gene expression of Cd-treated cells. Literature data on PLP2 are limited and fragmentary to date. It is known that the protein product, encoded by a 3.5-kb five-exon gene located on chromosome X, is a component of the endoplasmic reticulum with four trans-membrane domains, abundantly expressed in human colonic epithelium where it is supposed to control cell differentiation [18]. Interestingly, accumulation of PLP2 mRNA was observed during another differentiation event, i.e., that of the extraembryonic mesoderm in the early gastrula of rabbit embryo in coincidence with emerging morphogenetic activities at the posterior pole [19]. PLP2 protein is likely to multimerize and form ion channel due to the similarity of its hydropathy profile with that of a subunit of the H^+ vacuolar ATPase [20]. In addition, its overexpression was proven to be linked to enhanced migratory activity of C–C chemokine receptor-1-expressing cells in response to specific ligands, thus suggesting a role of plasma membrane-bound PLP2 in chemotactic processes [21].

Interestingly, some of us previously demonstrated the upregulation of *PLP2* in MDA-MB231 cells submitted to a different cytotoxic treatment, i.e., that, with 1 nM parathyroid hormone-related peptide fragment 38–94 [16]. Here, we provide first evidence that *PLP2* expression in MDA-MB231 cells can be also upregulated by "long-term" exposition to 5 μM $CdCl_2$ and that, in turn, such over-expression, although exerting no effect on the overall viability of the cell population, is linked to changes in the expression levels of stress response and caspase genes. Additional studies will be required to deeply understand the specific molecular mechanism through which PLP2 accomplishes its effect on Cd-exposed cells; however, the data presented here allow the following comments:

1. Transfection studies suggest that enhanced *PLP2* expression levels in response to $CdCl_2$ might be interpreted as a defence mechanism activated by cells in the attempt to prevent metal toxic effect by maintaining expression of *caspase-1*, *caspase-2*, and *caspase-9* at lower levels. Although examining expression at the mRNA level could provide evidence of differences that do not necessarily correspond to changes in enzyme activity, the observed transcriptional activation of such caspases following abrogation of *PLP2* upregulation suggests that expression of these members of the enzymatic family is specifically under the control of a PLP2-mediated mechanism whose nature is still to be determined.
2. Transfection studies also indicate that *PLP2* upregulation is in some way involved in restraining *hsc70* downregulation and *hsp27* over-expression, as a likely additional aspect of activation of cell defence mechanisms. It is known, in fact, that a decrease in *hsc70* expression may contribute to the lowering of cell protection against reactive oxygen species, in particular, as regards their activity on membrane lipid peroxidation [22]. In addition, in *Drosophila* cells, depletion of hsc70 has been proven to lead to upregulation of *hsp27* [23], and therefore the two events could be linked in PLP2 asODN-transfected cells. On the other hand, it is known that *hsp27* promoter contains an imperfect but functional estrogen-responsive element and that Cd is a metallo-estrogen, since it binds and activates steroid receptors [24, 25]; therefore, PLP2 might be involved in the counteraction of such activating role played by Cd on *hsp27* gene. Furthermore, Kindas-Mügge et al. [26] demonstrated the decrease of the proliferation rate of hsp27-overexpressing MDA-MB231 cells; thus, it can be hypothesized that PLP2-induced restraining effect on *hsp27* expression may be addressed to protect cell growth behavior from the toxic effect of the metal.
3. Utilizing a two-hybrid yeast system in KB epithelial cells, Wang et al. [27] had already reported that PLP2 is able to bind BAP31, another transmembrane polytopic protein of the endoplasmic reticulum. BAP31 is a substrate for cleavage by caspase-8, thereby producing the apoptosis-promoting form BAP20; on the other hand, PLP2 binding to intact BAP31 is supposed to render cells resistant to cytochrome c release from mitochondria and apoptosis induction via the death receptor (extrinsic) pathway, probably counteracting the enzymatic activity of caspase-8 on

BAP31 [28]. Interestingly, different from what was found for the other genes, asODN-mediated downregulation of *PLP2* was found to revert the upregulation of *caspase-8* previously observed in MDA-MB231 cells exposed to $CdCl_2$ [6]. Although the precise molecular mechanism is still to be understood, to our knowledge, this is the first evidence suggesting a correlation of PLP2 and the apoptosis-initiator caspase-8 also at gene expression level, additional to that already proposed at enzymatic activity level [27], thereby unveiling a novel positive interaction between the two transcriptional events which deserves further study.

4. Caspases form a complex enzymatic system whose members work together in a precisely controlled proteolytic cascade leading to auto- and mutual activation [e.g., 29]. The caspase members taken into account in the present paper are not all those involved in the onset of apoptosis, but only those whose gene expression levels have been proven to change following $CdCl_2$ treatment of MDA-MB231 cells [15]. In particular, caspase-1, caspase-2, and caspase-8 are involved in the death receptor pathway of apoptosis; caspase-9 is involved in the mitochondrial pathway, and both caspase-8 and caspase-9 activate caspase-3, caspase-6, and caspase-7 which act as apoptotic executioners. Therefore, taking such complexity into account, caution must be exercised in interpreting the data obtained by limited gene expression analyses, although they provide a good starting point for a more detailed study of Cd-mediated regulation of the caspase-cascade signalling system in malignant and immortalized epithelial breast cells.

In conclusion, the present work has expanded the knowledge on the biological effects of Cd–breast cancer cell interactions, indicating *PLP2* as a novel member of the list of Cd-regulated genes involved in the modulation of the pattern of transcriptional activities. Moreover, its differential expression observed also in the non-tumoral cell line here studied, i.e., HB2 immortalized cells from breast epithelium, suggests that promotion of *PLP2* upregulation is likely to be a widespread Cd-dependent phenomenon. Consequently, *PLP2* could be taken into consideration as an additional putative gene expression signature to serve in both prognostic and predictive assessments for the health risk of exposure to concentrations of Cd present in the occupational environment, although further validation in a statistically significant number of cell lines, both normal and pathological, as well as selected from different sources, will be required to this purpose.

Acknowledgments This work was supported by a grant from the University of Palermo, Italy (R.S. ex60%). We thank Ms. Mariapaola Ippolito for help in performing experiments during her laboratory internship. HB2 cells were courtesy of Cancer Research (UK).

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