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Cellular stress induces cap-independent alpha-enolase/MBP-1 translation

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ABSTRACT

Myc promoter-binding protein-1 (MBP-1) is a shorter protein variant of the glycolytic enzyme alpha-enolase. Although several lines of evidence indicate that MBP-1 acts as a tumor suppressor, the cellular mechanisms and signaling pathways underlying MBP-1 expression still remain largely elusive. To dissect these pathways, we used the SkBr3 breast cancer cell line and non-tumorigenic HEK293T cells ectopically overexpressing alpha-enolase/MBP-1. Here, we demonstrate that induced cell stresses promote MBP-1 expression through the AKT/PERK/eIF2α signaling axis. Our results contribute to shedding light on the molecular mechanisms underlying MBP-1 expression in non-tumorigenic and cancer cells.

1. Introduction

Many eukaryotic genes generate protein isoforms and variants with slightly different/overlapping functions or truncated proteins that have altered cellular localization and, consequently, alternative functions. The human ENO1 gene encodes the glycolitic enzyme alpha-enolase, which normally resides in the cytoplasm where it contributes to glycolysis, and a shorter protein variant, Myc promoter-binding protein-1 (MBP-1), which exerts its function in the nucleus. MBP-1 acts as a transcriptional repressor, and three direct gene targets, c-Myc, COX2 and ERBB2, have been identified so far [1–4]. Several reports indicate that MBP-1 acts as a putative oncosuppressor, negatively regulating cell proliferation or promoting cancer cell apoptosis when overexpressed in vitro [5,6]. Moreover, a role for MBP-1 in regulating tumor invasion and metastasis has been proposed for follicular thyroid carcinoma and gastric cancer, using in vitro and mouse xenograft models, respectively [7,3].

According to the well-known Warburg effect, cancer cells highly express almost all glycolytic enzymes, including alpha-enolase, a phenomenon which in toto confers a growth advantage, both in hypoxic and normal oxygen conditions, to the transformed cells [8]; in contrast, and consistent with its negative regulatory role on cell growth, the endogenous level of MBP-1 is very low compared to that of alpha-enolase, both in tumor and non-tumorigenic cell lines.

Miller's group reported that glucose concentration and hypoxia modulate MBP-1 expression in the MCF-7 breast cancer cell line [9,10], and it has been hypothesized that MBP-1 is also post-translationally regulated in a proteasome-dependent manner [11]. More recently, MBP-1 has been identified in *Arabidopsis thaliana* [12,13], and it was confirmed that the blockade of the ubiquitin-dependent degradation pathways contributes to elevating the stability of MBP-1 [12]. At present, no other studies

Abbreviations: MBP-1, Myc promoter-binding protein-1; 2-DG, 2-deoxyglucose; TG, thapsigargin; ER, endoplasmic reticulum; UPR, unfolded protein response; GRP78, glucose regulated protein 78; BEZ, NVP-BEZ235; LY, LY294002; EVE, everolimus; siRNA, small interfering RNA

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concerning the extracellular or cellular control of MBP-1 protein expression are available.

In breast cancer, we have shown that in almost all cases MBP-1 is easily and consistently detectable in the normal tissue surrounding the tumor, while in primary invasive ductal carcinoma (IDC) its expression varies and is correlated to patient outcome; ultimately, MBP-1 may be considered a predictor of good disease-free survival in this type of tumor [14].

The identification of novel biomarkers may improve the diagnosis, staging and prognostication of tumors and, in the case of breast cancer patients, due to the common resistance to systemic therapy, the demand for novel/personalized therapies is continuously increasing. To this end, we aimed at investigating the only partially-explored molecular mechanisms underlying MBP-1 expression.

Our own and other previous data support the existence of a single ENO1 gene transcript from which both alpha-enolase and MBP-1 arise through the use of alternative translation initiation sites [1,2]; one report indicates that a shorter variant transcript, originating from intron III of the gene, may contribute to MBP-1 expression in a variety of normal tissues and cancer cells [11]. In the present study, we provide further evidence that MBP-1 arises through the alternative translation of the transcript encoding alpha-enolase; furthermore, using a breast cancer cell line and a cellular model overexpressing alpha-enolase, we identified for the first time and partially dissected the stress signaling pathways promoting MBP-1 expression.

2. Material and methods

2.1. Reagents

2-Deoxy-D-glucose and everolimus were purchased from Sigma–Aldrich; thapsigargin and salubrinal were from Santa Cruz Biotechnology; NVP-BEZ235 and SC66 were from Cayman Chemicals; LY 294002 was from Cell Signaling Technologies.

2.2. Cell culture

SkBr3 and MCF-7 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and Human Embryonic Kidney 293T (HEK293T) cells were kindly provided by Dr. Sandra Pellegrini (Institut Pasteur, Paris, France). Cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM glutamine and 100 μg/ml penicillin/streptomycin (Sigma–Aldrich). For HEK293T cells, culture medium was supplemented with 1 mM sodium pyruvate (Sigma–Aldrich).

For treatment with inhibitors, SkBr3 or MCF-7 (7×10^5) and HEK293T (2×10^5) cells were cultured in DMEM low-glucose (1 g/l) (Sigma–Aldrich) plus 10% dialyzed FBS (Gibco, Life Technologies) on 6-well plates. The next day, cells were incubated in the same medium supplemented with the indicated amount of inhibitors; all treatments lasted 24 h.

2.3. Plasmid construction and stable transfection

The full-length Flag-Eno coding sequence was obtained by PCR with appropriate oligonucleotides, using the pFlag-Eno vector as template [15], and cloned into the pcDNA4/V5-His expression vector (Life Technologies); the resulting construct, pFlag-Eno-V5, was sequenced to verify the open reading frame. Stably transfected HEK293T cells expressing a multiple-epitope-tagged alpha-enolase were obtained by the calcium phosphate method, according to standard procedures, and subsequent selection with 200 μg/ml Zeocin (Life Technologies).

2.4. Cell extracts and immunoblotting

Total protein extracts were prepared as previously described [15]. Total proteins (20–30 μg) were separated on 9% polyacrylamide gels and then transferred onto nitrocellulose membrane. For analyses in Fig. 3, primary antibodies were revealed with horseradish peroxidase-linked secondary antibodies (GE Healthcare Life Sciences) and an ECL detection system (Thermo Scientific Pierce), as previously described [15]; for all the others blots the Odyssey infrared imaging system (LI-COR Biosciences) was used according to the manufacturer's instructions. Primary antibodies were: mouse Eno 276/3 and Eno 19/8 against alpha-enolase [14]; mouse anti-V5 (Life Technologies); rabbit anti-c-Myc, goat anti-PERK (Santa Cruz Biotechnology); rabbit anti-phospho-Akt, mouse anti-Akt, rabbit anti-phospho-PERK, rabbit anti-phospho-eIF2α, mouse anti-eIF2α (Cell Signaling Technologies); rabbit anti-ErbB2 (Proteintech), rabbit anti-Flag, rabbit anti-GRP78/BiP and mouse anti-β-actin (Sigma–Aldrich). Secondary antibodies were either conjugated to IRDye® 800CW (LI-COR) or Alexa Fluor 680 (Life Technologies).

2.5. Cell viability assay

Cells ($5–10 \times 10^3$) were cultured on 96-well plates in low-glucose medium for 24 h, then in fresh medium containing different inhibitor doses. MTS assays were performed using the CellTiter Aqueous OneSolution kit (Promega) according to the manufacturer's instructions. Cell viability was expressed as a percentage of the absorbance measured in the control cells. Results from three independent experiments, each performed in triplicate, were expressed as mean ± SD.

2.6. Small interfering RNA (siRNA) transfection

SkBr3 and HEK293T (6×10^4 and 2×10^4, respectively) cells were cultured in 12-well plates in low-glucose medium. After 24 h, the cells were transfected with pre-validated human PERK siRNA or control siRNA (Qiagen) at a final concentration of 5 nM, using HiPerFect siRNA transfection reagent according to the manufacturer's instructions (Qiagen). Cells were harvested and processed for immunoblotting 48 h after transfection.

3. Results and discussion

3.1. Glycolysis inhibitor 2-deoxyglucose and the ER stress-inducing drug thapsigargin promote the expression of MBP-1 in SkBr3 breast cancer cells

MBP-1 expression has been correlated to the glucose concentration in the culture medium; specifically, a low glucose concentration induces an increase of MBP-1 expression and a decrease of proliferation in MCF-7 human breast cancer cells [9]. This observation prompted us to investigate whether the glycolysis inhibitor 2-deoxyglucose (2-DG) may affect MBP-1 expression. The glucose analog 2-DG mimics glucose deprivation by suppressing the first step of glycolysis and may also induce endoplasmic reticulum (ER) stress and trigger the unfolded protein response (UPR) [16]. To investigate 2-DG effects on MBP-1 expression, we first treated a breast cancer cell line, SkBr3, with increasing concentrations of inhibitor in culture conditions close to the ones routinely used, i.e., simply lowering the glucose concentration in the medium (see Section 2). As previously reported, endogenous MBP-1

expression level is low in SkBr3 cells which carry an amplified ERBB2 gene, a recently recognized downstream target of MPB-1; however, these cells have been shown to retain a physiological response to MBP-1 overexpression [4]. The time of treatment and the optimal concentration of 2-DG were determined preliminarily by a small-scale experiment. MBP-1 protein expression was determined by immunoblotting and densitometric analysis data were normalized to β-actin levels. The 2-DG treatment upregulated MBP-1 in a dose-dependent manner, and the highest level of MBP-1 expression was observed after treatment with 5–15 mM 2-DG for 24 h, while no significant effect on alpha-enolase expression was detected (Fig. 1A and B).

In order to investigate whether ER stress is involved in MBP-1 expression, SkBr3 breast cancer cells were treated either with increasing concentrations of 2-DG or thapsigargin (TG). TG is a highly specific inhibitor of the sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+}-ATPase pump, which causes the elevation of intracellular calcium, ER stress and ultimately apoptosis if stress is not resolved through the UPR [17]. We observed a dose-dependent upregulation of MBP-1 expression following both 2-DG and TG treatments; consistent with the increased expression of MBP-1, its downstream targets ErbB2 and c-Myc proteins were downregulated (Fig. 1C). To correlate MBP-1 expression to known 2-DG or TG effects on cellular growth, SkBr3 cells viability was measured by MTS assay. Cell growth decreased by 60–80% when cells were treated with 5–15 mM 2-DG for 24 h, while treatment with TG induced an overall 40% reduction in cell viability (Fig. 2A and B).

It is known that AKT kinase plays a role in the control of cell proliferation and cell viability and is involved in the regulation of energy metabolism. Usually glucose deprivation results in the activation of AMPK and consequent inhibition of AKT phosphorylation; however, the activation of AKT kinase following 2-DG treatment has also been reported [18]. Therefore, we decided to investigate the relative AKT phosphorylation under our experimental conditions. As expected from the cell viability results, we did not observe an activation of AKT in 2-DG-treated SkBr3 cells, phosphorylation of S^{473} was reduced with respect to control cultures and, overall, the immunoblotting analysis showed an inverse association between AKT activity and MBP-1 expression (Fig. 1C, 2-DG). Cells treated with TG showed an even more significant reduction in phospho-AKT compared to the untreated control (Fig. 1C, TG).

To restore ER homeostasis, the UPR activates a number of protective mechanisms, including the transient attenuation of protein translation, the activation of selected gene expression, and the induction of molecular chaperones and folding enzymes. Among the actors of the UPR, PERK kinase, eIF2α, ATF4 and the ER chaperone glucose-regulated protein 78 (GRP78) are the downstream effectors of the event cascade which characterizes the response. Namely, as a response to stress, protein synthesis is mainly repressed through the blocking of cap-dependent mRNA translation, exerted by controlling the activity of the translation initiation factor eIF2α. Under stress conditions, the phosphorylation of eIF2α by PERK kinase inhibits the correct positioning of the ribosome on the mRNA start codon; consequently, cap-independent translation is activated to maintain the required levels of selected proteins (e.g., ATF4) and, ultimately, to resolve the stress or to induce apoptosis. In accordance with these data and confirming the effectiveness of the treatments, in SkBr3 cells treated with 2-DG or TG we found upregulated levels of active phospho-PERK (pT^{980}) and phospho-eIF2α, as well as an increase of ATF4 and GRP78 expression compared to the untreated control (Fig. 1C). The upregulation of UPR was dose-dependent and was associated with the induction of MBP-1 expression, suggesting a correlation between the two events.

3.2. The inhibition of PI3K-AKT signaling upregulates the downstream PERK/eIF2α pathway and MBP-1 expression

Since MBP-1 is generated by the alternative translation of ENO1 mRNA, the results of 2-DG and TG treatments on SkBr3 cells strongly suggested that the observed upregulation of MBP-1 expression under ER stress conditions might be regulated by a cap-independent mechanism switched on following eIF2α inactivation.

Recently, a link between AKT kinase activity and the phosphorylation/inactivation of eIF2α has been found. In physiological conditions, AKT-mediated phosphorylation of PERK kinase in T^{799} maintains low activity; PERK, in this inactive form, does not phosphorylate and consequently inactivate eIF2α, which in the unphosphorylated form exerts its routine role in translation initiation [19]. The treatment of cells with pharmacological inhibitors of PI3K-AKT mimics ER stress induction, resulting in the downregulation of phospho-AKT, the upregulation of active PERK kinase (autophosphorylation in T^{980}) and inactive phospho-eIF2α [19].

To experimentally support the hypothesis of a correlation between the active/inactive state of AKT, the PERK-mediated phosphorylation of eIF2α, and MBP-1 expression, SkBr3 breast cancer cells were first treated with the AKT inhibitor, NVP-BEZ235 (BEZ). This molecule is a dual inhibitor that blocks the activity of both PI3K and mTOR signaling, competing for binding with ATP [20]. As expected, the treatment with this inhibitor induced a decrease of phospho-AKT, while phospho-PERK, phospho-eIF2α, ATF4 and MBP-1 expression were upregulated (Fig. 1D). As a control, we evaluated the effects on cell growth; in our experimental conditions, BEZ treatment decreased cell viability of SkBr3 cells by 35–40% (Fig. 2C). To further investigate whether the PERK-mediated translation control downstream of AKT is involved in the upregulation of MBP-1 expression, we used two inhibitors of the PI3K/AKT/mTOR pathway, LY294002 (LY) and everolimus (EVE). LY specifically inhibits the upstream PI3K, and EVE blocks the downstream mTOR complex. Consistent with these selective action mechanisms, LY treatment induced a significant decrease of phospho-AKT in SkBr3 cells, while treatment with EVE did not have such an effect (Fig. 1D). As a control of EVE treatment, we monitored the decrease of phospho-p70S6K, which is a downstream effector of AKT/mTOR (not shown). In conclusion, both treatments with LY and BEZ resulted in an increased phosphorylation/activation of PERK and phosphorylation/inactivation of eIF2α, and a concomitant upregulation of MBP-1 expression.

These results were in agreement with both the previously reported data regarding the control exerted by AKT-PERK signaling on eIF2α activity [19] and our hypothesis of a downstream effect on MBP-1 translation. To further confirm the putative association of the AKT/PERK/eIF2α signaling pathway with MBP-1 expression, we used a novel AKT inhibitor, SC66. This inhibitor has a dual function: it facilitates ubiquitination and degradation of AKT and inhibits its activity by interfering with the binding of the AKT PH domain to PIP3 [21]. In SkBr3 cells treated with SC66 inhibitor, the blocking/degradation of AKT resulted in an increased phosphorylation/activation of PERK kinase, leading to the inactivation of eIF2α by phosphorylation in S^{51}, an increased expression of the downstream target ATF4, and a concomitant upregulation of MBP-1 (Fig. 1D). Cell viability was reduced by 60–75% (Fig. 2D). Comparable outcomes were obtained in MCF-7 breast cancer cells following the same treatments (Fig. 1E), and in no case alpha-enolase expression was significantly affected. Overall, these results strongly support the hypothesis that ENO1 mRNA alternative translation may be controlled by the AKT/PERK/eIF2α pathway.

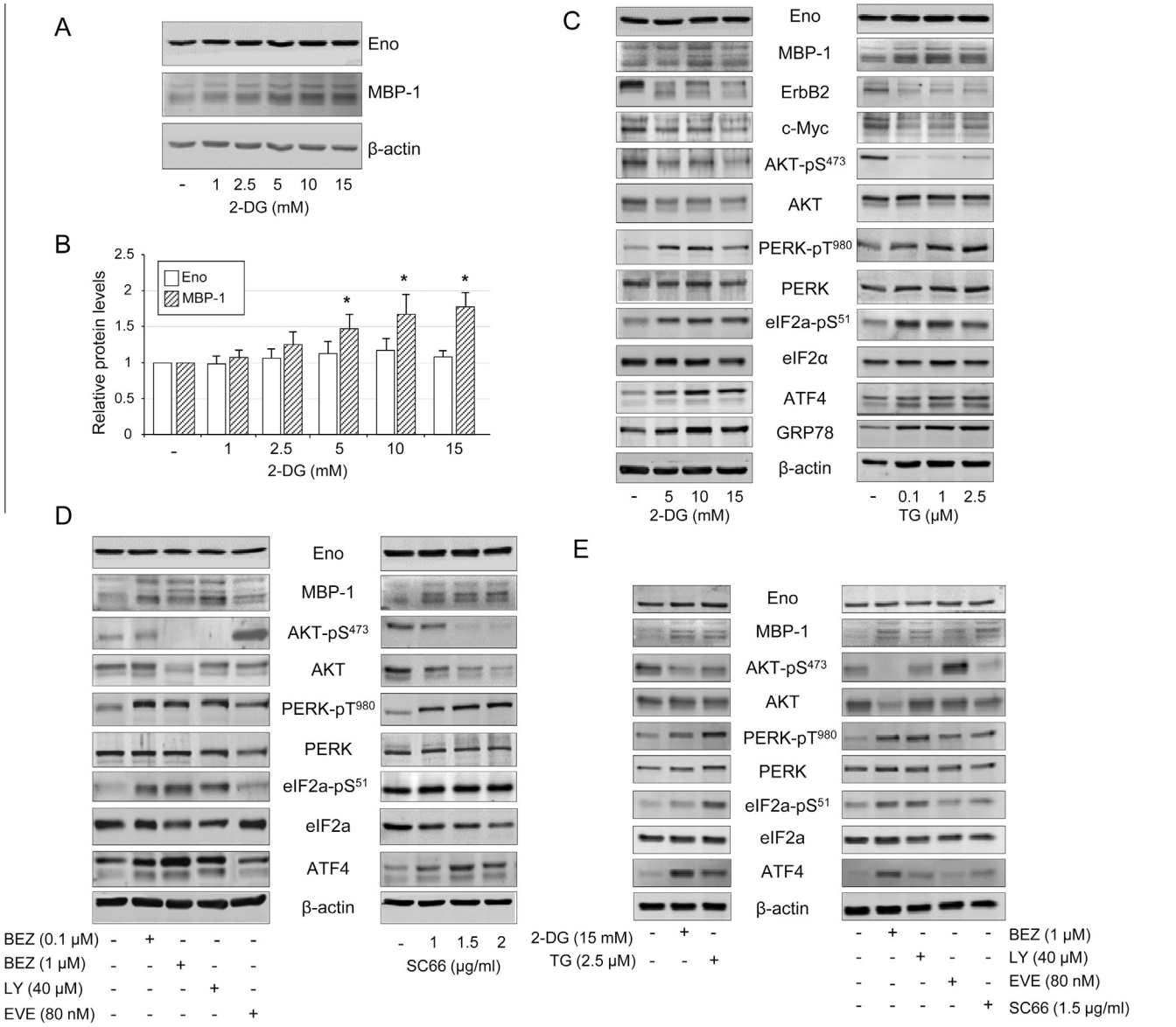

Fig. 1. The treatment of breast cancer cells with ER stress inducers and PI3K-AKT signaling inhibitors results in the activation of the PERK/eIF2α pathway and increased MBP-1 expression. (A) To set experimental conditions, SkBr3 cells were treated with the indicated concentrations of 2-DG for 24 h, alpha-enolase (Eno) and MBP-1 protein levels were determined by immunoblotting. (B) Data were normalized to β-actin and expressed relative to the untreated control (−). Each data point is the average of at least three independent experiments, bars represent standard deviation ($^{*}P < 0.005$). (C) After a 24-h treatment with 2-DG or TG at the indicated concentrations, the expression levels of MBP-1, downstream targets ErbB2 and c-Myc, and phospho-AKT, phospho-PERK, pospho-eIF2α, ATF4 and GRP78 proteins were determined by immunoblotting. (D) SkBr3 cells were treated with the indicated concentrations of NVP-BEZ235 (BEZ), LY294002 (LY), everolimus (EVE) or SC66 for 24 h. The expression of MBP-1, phospho-AKT and ER stress proteins was determined by immunoblotting. (E) MCF-7 cells were subjected to the indicated treatments for 24 h; as for SkBr3 cells, MBP-1 expression was upregulated upon treatment with 2-DG, TG, BEZ, LY and SC66, but not in the presence of EVE. β-Actin is shown as a control of the total proteins loaded per lane. Results are representative of three independent experiments with comparable outcomes.

3.3. The use of a cellular model ectopically expressing alpha–enolase/MBP-1 unravels the signaling pathways underlying MBP-1 expression

To confirm the results obtained in SkBr3 cells and to demonstrate an ER stress-mediated control of MBP-1 expression, we utilized a HEK293T cell clone, which stably expresses recombinant human alpha-enolase/MBP-1 proteins.

Cells were obtained by the stable transfection of an expression vector carrying an alpha-enolase cDNA with additional epitopes: a Flag-Tag at the NH2-terminal and a V5-Tag plus six-histidine at the COOH-terminal. 293-T/Eno-Flag-V5 cells express both the recombinant multiple-epitope-tagged alpha-enolase and the double-tagged MBP-1-V5 protein generated by the alternative translation of the recombinant mRNA. The ectopically expressed proteins are easily distinguished by using specific anti-Tag antibodies (anti-Flag and anti-V5, Fig. 3A and B), while the use of monoclonal antibodies with known antigen specificity allows for the simultaneous detection of endogenous and exogenous alpha-enolase/MBP-1 proteins (anti-Eno 276/3 and anti-Eno 19/8, Fig. 3C). Although wild type HEK293T cells express almost undetectable levels of endogenous MBP-1, the treatment of the stably transfected cells with 2-DG resulted in a dose-dependent increased expression of exogenous MBP-1-V5, confirming once more the origin of MBP-1 from the alternative translation of full-length alpha-enolase mRNA (Fig. 3D).

We then subjected the cells carrying the recombinant alpha-enolase/MBP-1 proteins to the same stimuli utilized on SkBr3 cells and associated the expression level of ectopic

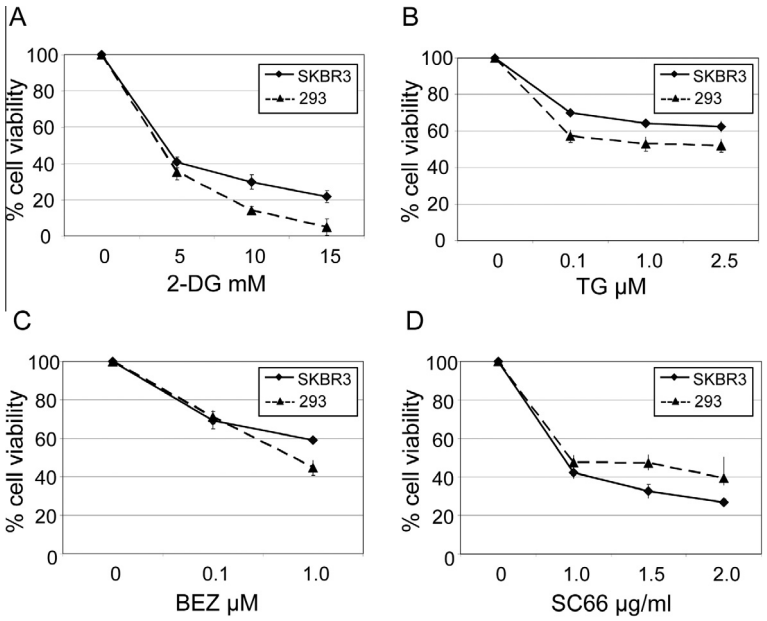

Fig. 2. Cell viability upon treatments was assessed by MTS assay. SkBr3 or 293-T/Eno-Flag-V5 (293) cells were treated with 2-DG (A), TG (B), BEZ (C) or SC66 (D) at the indicated concentrations for 24 h. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was analyzed in triplicate.

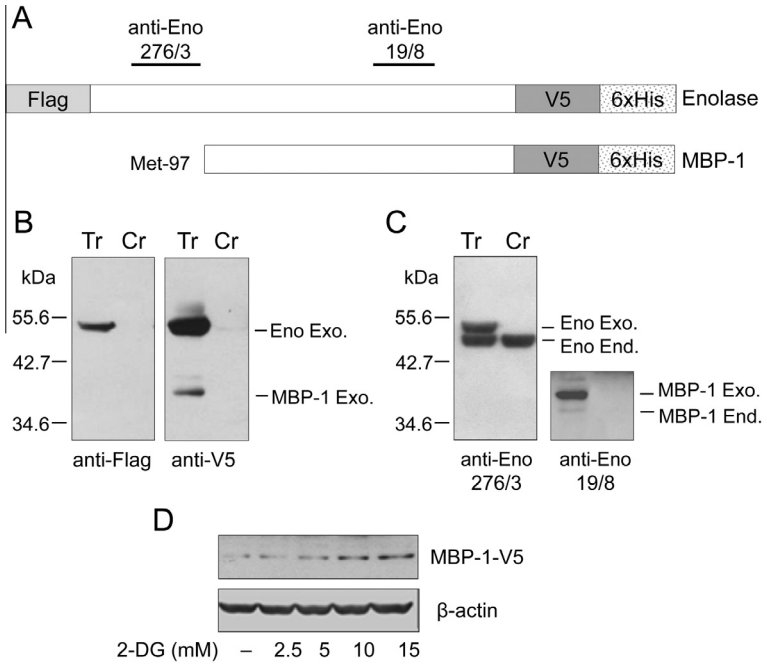

Fig. 3. 293-T/Eno-Flag-V5 cells ectopically expressing recombinant alpha-enolase/MBP-1 proteins respond to 2-DG by upregulating exogenous MBP-1 protein. (A) Schematic representation of the epitope-tagged alpha-enolase construct, the location of Flag, V5 and 6xHis tags, and of protein regions containing the epitopes recognized by anti-Eno 276/3 and anti-Eno 19/8 monoclonal antibodies are indicated. (B and C) Analysis of endogenous (End.) and exogenous (Exo.) alpha-enolase/MBP-1 proteins by immunoblotting. Total proteins from stably transfected cells (Tr) and mock transfected cells (Cr) were separated on the same gels and probed with the indicated antibodies. The blot in (C) was first probed with anti-Eno 276/3 then re-probed with anti-Eno 19/8 antibody and exposed to film for 5 times longer than the others. (D) Cells were treated with the indicated concentrations of 2-DG for 24 h and MBP-1-V5 protein levels were determined by immunoblotting. β-Actin is shown as a control of the total proteins loaded per lane.

MBP-1-V5 with the pathways previously characterized. Overall, the cellular model responded to the different treatments, such as the ones leading to an ER stress response (2-DG and TG, Fig. 4A) and the ones interfering with the PI3K/AKT/mTOR pathway (BEZ, LY, EVE and SC66, Fig. 4B), with an increase of MBP-1-V5 protein, as previously observed in SkBr3 and MCF-7 cells. Cell viability data confirmed the effectiveness of the treatments (Fig. 2A–D).

Briefly, all treatments, except the one with EVE induced a decrease in phospho-AKT, the upregulation of phospho-eIF2α, and the increased expression of ATF4 and MBP-1. In conclusion, the overexpression of MBP-1-V5 was consistently driven by the inhibition of the PI3K-AKT signaling pathway and by ER stress activation through the AKT/PERK/eIF2α pathway.

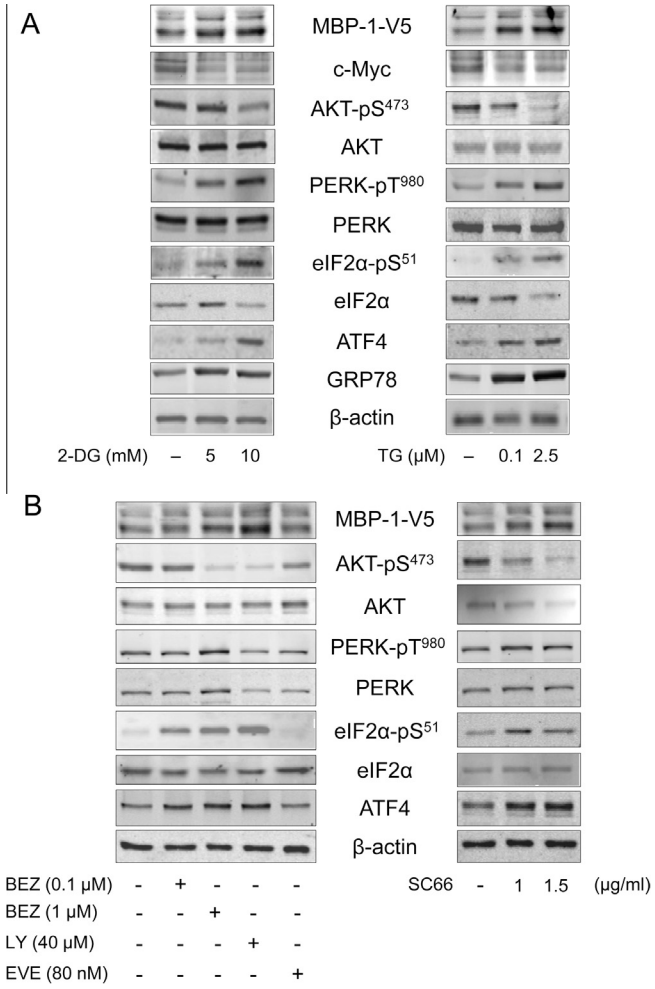

Fig. 4. The treatment of 293-T/Eno-Flag-V5 cells with ER stress-inducing drugs or AKT inhibitors leads to an increase of the recombinant MBP-1-V5 protein. Cells were treated with the indicated concentrations of 2-DG or TG (A) or NVP-BEZ235 (BEZ), LY294002 (LY), everolimus (EVE) or SC66 for 24 h (B). The expression of MBP-1-V5, downstream target c-Myc, phospho-AKT and ER stress proteins was determined by immunoblotting. β-Actin is shown as a control of the total protein loaded per lane. ER stress response and MBP-1-V5 expression were upregulated upon all treatments except EVE. Results are representative of three independent experiments with comparable outcomes.

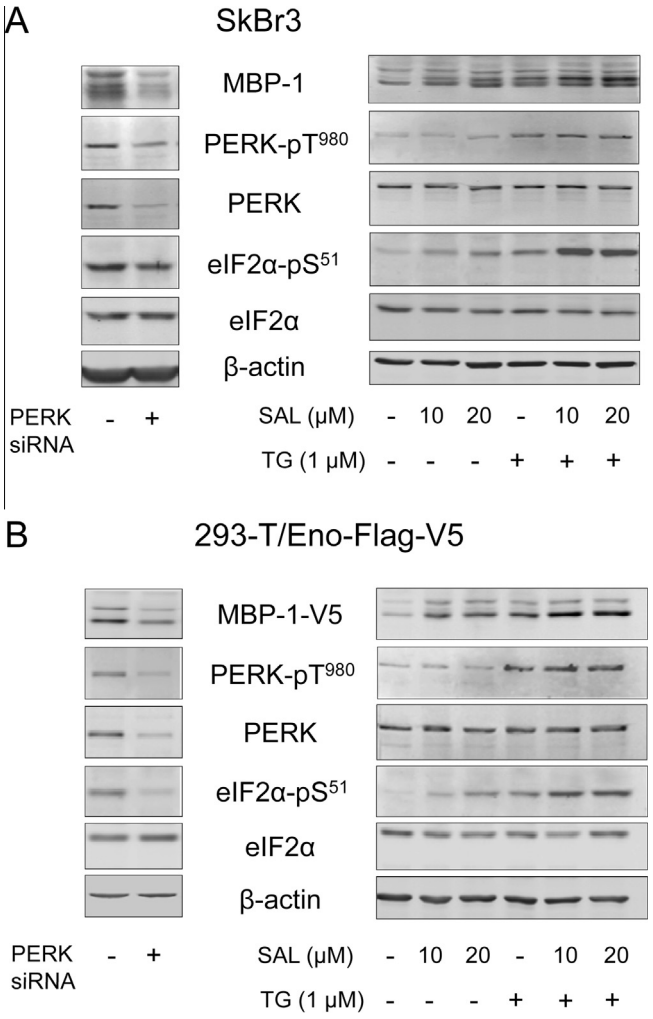

Fig. 5. The knockdown of PERK negatively affects MBP-1 and MBP-1-V5 expression in SkBr3 and 293-T/Eno-Flag-V5 cells, while Salubrinal treatment upregulates both MBP-1 proteins. Cells were treated with PERK-specific double stranded siRNA (+) or unrelated siRNA (−) (A, B, inner left) or with the indicated concentrations of Salubrinal (SAL) (A, B, inner right). The expression of MBP-1, MBP-1-V5, and ER stress proteins phospho-PERK and phospho-eIF2α was determined by immunoblotting. β-Actin is shown as a control of the total proteins loaded per lane. Results are representative of three independent experiments with comparable outcomes.

It is noteworthy that in 293-T/Eno-Flag-V5 cells MBP-1-V5 appears as a discrete band, while in SkBr3 cells MBP-1 protein appears as two/three closely migrating bands of slightly different apparent molecular weight, probably due to cell-type specific post-translational modifications.

3.4. MBP-1 expression is downregulated by PERK knockdown and enhanced by Salubrinal

To confirm the hypothesized central role played by PERK kinase downstream of AKT in MBP-1 expression, we silenced PERK by siRNA transfection both in SkBr3 and 293-T/Eno-Flag-V5 cells. The knockdown of PERK resulted in the downregulation of phospho-eIF2α and, ultimately, in a lower expression of MBP-1 and MBP-1-V5 proteins (Fig. 5A and B).

Finally, to further rule out the involvement of ER stress response signaling in the cap-independent translation underlying MBP-1 expression, we treated SkBr3 and 293-T/Eno-Flag-V5 cells with Salubrinal, a selective phosphatase inhibitor that prevents the dephosphorylation of phospho-eIF2α. The treatment with Salubrinal resulted in the upregulation of phospho-eIF2α and increased expression of MBP-1 and MBP-1-V5 proteins, without affecting the phosphorylation of PERK in T^{980}. Conversely, simultaneous treatment with TG and Salubrinal had a synergistic effect, increasing the levels of active phospho-PERK and inactive phospho-eIF2α and, ultimately, of MBP-1 and MBP-1-V5 proteins (Fig. 5A and B).

Collectively, our data indicate that the stress-induced inactivation of AKT kinase and the activation of the cellular response mediated by active phospho-PERK and inactive phospho-eIF2α induces MBP-1 expression, strongly suggesting that MBP-1 belongs to the group of proteins selectively expressed by a cap-independent mechanism as a consequence of cell response to stress conditions. These data contribute to shedding light on the signal-activation mechanisms leading to MBP-1 expression and may suggest novel therapeutic approaches to counteract the loss of MBP-1 in breast cancers.

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