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Protein kinase C-eta regulates Mcl-1 level via ERK1

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ABSTRACT

Protein kinase C (PKC)-eta (PKCη) is a member of the novel category of PKC family. It is overexpressed in breast cancer and was shown to inhibit apoptosis and contribute to chemoresistance. Since the anti-apoptotic Bcl-2 family protein myeloid cell leukemia-1 (Mcl-1) plays an important role in breast cancer cell survival and chemoresistance, we investigated if PKCη regulates Mcl-1 level. Silencing of PKCη decreased Mcl-1 in several breast cancer cells, including MCF-7 and T47D cells. PKCη depletion had no effect on *MCL1* mRNA but the decrease in Mcl-1 by PKCη knockdown was blocked by proteasomal inhibitors, such as MG132 and lactacystin. Moreover, knockdown of Mule (Mcl-1 ubiquitin ligase) prevented Mcl-1 downregulation caused by PKCη deficiency. Overexpression of catalytically-active Akt or knockdown of glycogen synthase kinase-3 (GSK3)-β, a substrate for Akt, had little effect on Mcl-1 downregulation caused by PKCη silencing. However, knockdown of PKCη but not PKCα, -δ or -ε caused a significant decrease in ERK (extracellular signal-regulated kinase) phosphorylation. Knockdown of ERK1 but not ERK2 decreased Mcl-1 level, and the decrease in Mcl-1 caused by PKCη knockdown was restored by ERK1 overexpression. These results suggest that PKCη utilizes the ERK signaling pathway to protect against ubiquitin-mediated proteasomal degradation of Mcl-1.

1. Introduction

Protein kinase C is a multigene family of serine/threonine kinases that regulate diverse cellular processes such as cell proliferation, differentiation and cell death [1,2]. On the basis of their structural properties and sensitivity to cofactors, the PKC family is categorized into conventional (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical PKCs (ζ, λ/ι) [2,3]. While conventional PKCs require diacylglycerol (DAG) and Ca^{2+} for their activity, novel PKCs are dependent on DAG but insensitive to Ca^{2+}, and atypical PKCs are insensitive to both Ca^{2+} and DAG [2,4]. PKCη appears to be a unique member of the novel PKC isozyme family [5]. While prolonged treatment with tumor promoting phorbol esters leads to downregulation of conventional and novel PKCs, PKCη either resists downregulation or is upregulated by phorbol esters [5–7].

The PKC isozymes may exhibit similar, distinct or even opposite roles in regulating cell survival and cell death [8,9]. For example, while PKCδ is believed to be pro-apoptotic [10–12] PKCε is considered anti-apoptotic [13–15] although the function of the same PKC isozyme may vary depending on the cellular context [8–11,13]. PKCη has been implicated in both tumor promotion and tumor suppression [5]. While mice lacking PKCη were more susceptible to tumor formation in a two-stage carcinogenesis model [16], it was also associated with several cancers, including renal cell carcinoma [17], glioblastoma [18], non-small cell carcinoma [19], acute myeloid leukemia [20] and breast cancer [21,22]. In addition, PKCη was shown to inhibit apoptosis and contribute to chemoresistance in several cancers, including breast cancer [23–27].

The Bcl-2 family proteins play critical roles in regulating cell death by apoptosis [28]. The Bcl-2 family consists of both anti-apoptotic (e.g., Bcl-2, Bcl-xl and Mcl-1) and pro-apoptotic (e.g., Bax, BAD, Bim and Bid) members [28]. Several PKC isozymes were shown to regulate Bcl-2 family proteins [13,29]. For example, overexpression of PKCα caused phosphorylation of Bcl-2 at Ser-70, which led to stabilization of Bcl-2 and suppression of apoptosis [30]. On the other hand, PKCδ was shown to promote cell survival via stabilization of anti-apoptotic Mcl-1 [31] and inhibit cell survival through the activation of pro-apoptotic Bax and Bak [32]. In addition, the catalytic fragment of PKCδ was shown to phosphorylate Mcl-1 and target it for degradation, thus facilitating cell death [33]. We and others have shown that the anti-apoptotic function of PKCε is associated with increased expression of Bcl-2 or down-regulation/inhibition of pro-apoptotic Bcl-2 family proteins [34–41].

PKCη shares greatest homology with PKCε [42] but there have been no reports on the involvement of PKCη in the regulation of the Bcl-2

Abbreviations: DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase-3; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia-1; Mule, Mcl-1 ubiquitin ligase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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family proteins. In the present study, we show that PKCη acts via ERK1 to protect Mcl-1 against degradation via the ubiquitin proteasome pathway involving Mcl-1 ubiquitin ligase Mule.

2. Materials and methods

2.1. Materials

MG-132 and Lactacystin were purchased from Calbiochem (San Diego, CA). TPA was purchased from Alexis Biochemicals (San Diego, CA). Polyclonal antibodies to PKCη, PKCδ, PKCε, Mcl-1 and monoclonal antibodies to Bcl-2, Bcl-xL, Mcl-1 and GSK3α/β were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies against Mcl-1, Mule and phospho-ERK were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal antibody to PKCα was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit monoclonal antibody against ERK1 was purchased from Abcam Inc. (Cambridge, MA). Monoclonal antibodies against actin and tubulin were obtained from Sigma (St. Louis, MO). siRNAs were obtained from Dharmacon (Lafayette, CO) and Qiagen (Germantown, MD) and Lipofectamine RNAiMax transfection reagent was obtained from Invitrogen (Carlsbad, CA). Horseradish-peroxidase-conjugated donkey anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Enhanced chemiluminescence detection kit was purchased from Amersham (Arlington Heights, IL).

2.2. Cell culture

MCF-7 and T47D cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37 °C with 95% air and 5% CO_2.

2.3. Transfection

Control non-targeting siRNA, SMARTpool siRNA or individual siRNAs were introduced into MCF-7 or T47D cells using Lipofectamine RNAiMax and manufacturer's protocol. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis.

2.4. Reverse transcriptase PCR

MCF-7 and T47D cells were transfected with control, non-targeting siRNA or siRNA against PKCη using manufacturer's protocol. Total RNA was extracted 48 h post-transfection using TRI Reagent from Molecular Research Center, Inc. (Cincinnati, OH). cDNA was synthesized using random primers and Improm II reverse transcriptase from Promega (Madison, WI). PCR amplification of cDNA was performed using Promega PCR Master Mix (Madison, WI) and primers for PKCη, Mcl-1 and GAPDH. The sequences of forward and reverse PKCη primers were 5′-ATGCGGTGGAACTTGCCA-3′ and 5′-CGTGACCACAGAGCATCTCA TAGA-3′ respectively. The sequences of forward and reverse Mcl-1 primers were 5′- CTTACGACGGGTTGGG-3′ and 5′-GGTTCGATG CAGCTTTCTTGG-3′ respectively. The sequences of the forward and reverse GAPDH primers were 5′- ACTGTGGTCATGAGTCCTTC-3′ and 5′- GAGCGAGATCCCTCCAA -3′ respectively. Cycle conditions for all PCRs were set up for 25 cycles as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

2.5. Immunoblot analysis

Cells were lysed in extraction buffer containing 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EGTA, 1 mM EDTA, 1.0% Triton X-100, 0.5% Nonidet-40, 10 mM β-glycerophosphate, protease inhibitor cocktail and phosphatase inhibitor cocktail. Equivalent amounts of total proteins (15–25 μg) were electrophoresed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA). The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. The blots were probed with actin or tubulin to control for equal loading.

2.6. Statistical analysis

The intensities of immunoreactive proteins were quantified using ImageJ software (National Institutes of Health). Statistical significance was determined by paired Student's *t*-test using Microsoft Excel. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of PKCη on Bcl-2 family proteins

PKCη has been implicated in promoting survival of breast cancer cells [5]. Several members of the PKC family were shown to regulate Bcl-2 family proteins [3,43] but the role of PKCη in the regulation of the Bcl-2 family is yet unknown. We therefore examined the effect of PKCη depletion on the Bcl-2 family proteins. Fig. 1A shows that knockdown of PKCη decreased the level of the anti-apoptotic Bcl-2 family protein Mcl-1 but increased the level of Bcl-2 with little change in Bcl-xL. We extended our study to another breast cancer cell line. Like MCF-7 cells, T47D breast cancer cells also express Mcl-1 and Bcl-xl but Bcl-2 is not detectable. Depletion of PKCη also decreased Mcl-1 in T47D cells (Fig. 1C). Densitometric quantification of Mcl-1 levels upon PKCη depletion from several independent experiments demonstrated a significant decrease in Mcl-1 upon PKCη depletion in both MCF-7 and T47D cells (Fig. 1B & D). Fig. 1E shows that knockdown of PKCη in T47D cells by several different siRNAs was effective in inducing Mcl-1 downregulation.

We then compared the effects of several PKC isozymes on Mcl-1 levels. Fig. 2A shows that knockdown of both PKCδ and PKCη decreased Mcl-1 levels although based on several independent experiments, the effect of PKCη knockdown on Mcl-1 downregulation was greater (Fig. 2B). In contrast, knockdown of PKCε decreased Bcl-2 but not Mcl-1 (Fig. 2A).

3.2. Effect of PKCη depletion on Mcl-1 mRNA

Mcl-1 can be regulated both at the transcriptional and the post-transcriptional level [44,45]. To determine whether PKCη regulates Mcl-1 expression at the mRNA level, we transfected cells with control non-targeting or PKCη siRNA and examined the mRNA expression of *MCL1* (encodes Mcl-1) by reverse-transcriptase PCR. As shown in Fig. 3, PKCη depletion did not alter *MCL1* mRNA, suggesting that PKCη does not regulate *Mcl-1* at the transcriptional level.

3.3. Effect of PKCη depletion on the proteasomal degradation of Mcl-1

Mcl-1 has a short half-life since it is rapidly degraded via the ubiquitin proteasome-mediated pathway [44–46]. Therefore, we examined the effect of proteasome inhibitors on PKCη knockdown-mediated downregulation of Mcl-1. Fig. 4A shows that the treatment of MCF-7 cells with the proteasome inhibitors MG-132 or lactacystin enhanced basal Mcl-1 level substantially. While knockdown of PKCη decreased Mcl-1 level by > 2-fold, MG132 and Lactacystin attenuated PKCη knockdown-mediated downregulation of Mcl-1 to 1.2- and 1.5-fold, respectively (Fig. 4B).

Since Mule was identified as Mcl-1 ubiquitin ligase [46], we examined the effect of Mule knockdown on Mcl-1 downregulation. As shown in Fig. 4C, knockdown of Mule using two different siRNAs enhanced basal Mcl-1 level and prevented Mcl-1 downregulation caused by PKCη depletion. These results suggest that PKCη prevents proteasomal degradation of Mcl-1.