FEN ve MÜHENDİSLİK BİLİMLERİ DERGİSİ

Vol: 7 No: 1 Year: 2025 Araştırma Makalesi/Research Article e-ISSN: 2667-7989

https://doi.org/10.47112/neufmbd.2025.74

Metastatik Meme Kanseri Hücrelerinde EGF Uyarımına Bağlı Olarak PKA, AKT(PKB) ve PKC Hedefli AKT Substratlarının Fosforilasyon Düzeylerinin Değişimi

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Makale Bilgisi

Geliş Tarihi: 31.08.2024
Kabul Tarihi: 20.11.2024
Yayın Tarihi: 30.04.2025

Anahtar Kelimeler:
PI3K/AKT Yolağı,
AKT Substratı,
PKA, PKB ve PKC
inhibisyonu.

ÖZET

Protein kinaz B (PKB/AKT), hücre metabolizması, büyüme, çoğalma ve tümör invazyonu dahil olmak üzere çok çeşitli fizyolojik ve patolojik süreçleri düzenleyen fosfatidilinositol-3-kinaz (PI3K)/AKT sinyal yolağının temel düzenleyicisidir. AKT enzimi, EGF gibi hücresel uyarıcı moleküller tarafından aktive edilebilen bir serin/treonin kinazdır. Aktive AKT enzimi, hedef substratlarını RxRxxS/T konsensus dizisine bağlanarak fosforile eder. Bazı AKT hedefleri, farklı serin/treonin kinazlar tarafından benzer rezidulardan fosforile edilebilmektedir. Bu substratlar üzerinde yapılan birçok çalışma, sinyal yolakları arası etkileşimi sağlayan ortak moleküllerin ve yeni terapötik hedeflerin belirlenmesi için değerli bilgiler sunmaktadır. Bu bağlamda söz konusu çalışmada, EGF uyarımına bağlı olarak MDA-MB-231 üçlü negatif metastatik meme kanseri hücrelerinde, AKT substrat fosforilasyonlarını mimik eden PKA, PKB(AKT) ve PKC hedefli yeni moleküllerin belirlenmesi amaçlanmıştır. AKT substratlarının fosforilasyon düzeyleri Western blot analizi ile değerlendirildi. Elde edilen sonuçlara göre, PKA, PKB ve PKC inhibitörlerinin varlığında ve EGF uyarımı ile birlikte 30 kDa ağırlığında bir fosfo-proteininin (pp30), AKT substrat fosforilasyon düzeyinin değiştiği saptandı. EGF uyarımına bağlı olarak, beklendiği üzere AKT substratlarının fosforilasyon düzeyleri artarken, inhibitörler bu fosforilasyonları baskılamaktadır. Dikkat çekici bir şekilde, PKC inhibitörü varlığında pp30'un fosforilasyon düzeyinin, EGF uyarımına rağmen kontrole kıyasla azaldığı belirlendi. Bu sonuç, pp30'un hücresel regülasyonunun PKC tarafından AKT ile aynı bölgeden gerçekleşebileceğini ortaya koymaktadır. Çalışmamızdaki metodoloji, AKT ile PKC sinyal yolakları arasındaki etkileşimde rolü olabilecek yeni potansiyel düzenleyicilerin tanımlanabilmesinin mümkün olduğunu göstermektedir. Ancak, bu etkileşimin ne yönde olduğunu açıklayabilmek için ileri çalışmalara ihtiyaç vardır.

Changes in Phosphorylation Levels of PKA, AKT(PKB) and PKC Targeted AKT Substrates Upon EGF Stimulation in Metastatic Breast Cancer Cells

Article Info

Received: 31.08.2024
Accepted: 20.11.2024
Published: 30.04.2025

Keywords:
PI3K/AKT pathway,
AKT substrate,
PKA, PKB and PKC
inhibition.

ABSTRACT

Protein kinase B (PKB/AKT) is a crucial regulator of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, which regulates a wide range of physiological and pathological processes, including cell metabolism, growth, proliferation, and tumor invasion. The AKT enzyme is a serine/threonine kinase that can be activated by cellular stimuli such as EGF. Once activated, the AKT enzyme phosphorylates its target substrates by binding to the RxRxxS/T consensus sequence. Some AKT targets can be phosphorylated at similar residues by different serine/threonine kinases. Numerous studies on these substrates provide valuable insights into common molecules facilitating cross-talk between signaling pathways and the identification of new therapeutic targets. In this study, we aimed to identify novel molecules that target PKA, PKB (AKT), and PKC and mimic AKT substrate phosphorylation in EGF-stimulated MDA-MB-231 triple-negative metastatic breast cancer cells. Phosphorylation levels of AKT substrates were assessed using Western blot analysis. According to the results, the phosphorylation level of a 30 kDa phosphoprotein (pp30) varied in the presence of PKA, PKB, and PKC inhibitors, as well as upon EGF stimulation. As expected, phosphorylation levels of AKT substrates increased with EGF stimulation, while the inhibitors suppressed these phosphorylations. Interestingly, in the presence of the PKC inhibitor, the phosphorylation level of pp30 was found to be decreased compared to the control, despite EGF stimulation. This suggests that cellular regulation of pp30 by PKC might occur through the same region as AKT. Our methodology demonstrates the potential to identify new regulators that may play a role in the interaction between AKT and PKC signaling pathways. However, further studies are required to elucidate the specifics of this interaction.

To cite this article:
Özcan Türkmen, M., Pehlivanoğlu, Ş. & Pehlivanoğlu, S. (2025). Changes in phosphorylation levels of PKA, AKT(PKB) and PKC targeted AKT substrates upon EGF stimulation in metastatic breast cancer cells. *Necmettin Erbakan University Journal of Science and Engineering, 7*(1), 48-55. https://doi.org/10.47112/neufmbd.2025.74

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INTRODUCTION

Cancer is the second leading cause of death globally after ischemic heart disease [1-2]. According to the Global Cancer Statistics 2022, there were nearly 20 million new cancer cases and 9.7 million deaths due to cancer in that year [3]. In women, breast cancer was the most common, while in men, lung cancer was the most frequent, both in terms of cases and deaths in 2022 [3]. Breast cancer is the most frequently diagnosed cancer and one of the main causes of cancer-related deaths in women worldwide [4-5]. This cancer is characterized by the abnormal growth and proliferation of cells originating in the breast tissue [6]. It is a metastatic cancer and often spreads to distant organs such as the liver, bone, lung, and brain, which primarily accounts for its incurability [7]. Numerous risk factors associated with breast cancer have been established, including older age, female gender, early menarche, late menopause, positive family history, long-term hormone replacement therapy, a history of radiation therapy to the chest, and *BRCA1/2* gene mutations [5, 8-9]. The prognosis of breast cancer and the patient's response to distinct types of therapies vary among subtypes of this heterogeneous disease [10]. These subtypes are typically classified into four categories depending on the immunohistochemical expression of specific biomarkers: estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), human epidermal growth factor receptor 2-positive (HER2+), and triple-negative breast cancer (TNBC), which is characterized by the absence of expression of any of these receptors [11]. HER2-positive and endocrine-sensitive diseases can be treated with targeted therapies, whereas chemotherapy remains the primary treatment for TNBC [12]. The effectiveness of targeted treatments is limited by primary or secondary resistance [12]. Increasing evidence indicates that the active phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway plays a key role in treatment resistance and disease progression [12].

The persistence of proliferation stimulation is among the distinguishing features of cancer and many genes and proteins, especially kinases and kinase receptors, play an active role in this process. [13]. One member of the protein kinase family is protein kinase B (PKB) which is also known as AKT [14]. PKB/AKT is a serine-threonine kinase which is a member of the AGC (protein kinase A, PKC, PKG) kinase superfamily [14-15]. It has three isoforms (AKT1, AKT2 and AKT3) in mammals and their kinase domains have extensive homology to these AGC kinases [16]. AKT is activated by growth factors, cytokines and other cellular stimulatory molecules and activated AKT phosphorylates a large number of substrates which play roles in many cellular signaling pathways [16]. These pathways are involved in several important cellular processes including cell survival, proliferation, cell size in response to nutrient availability, growth, metabolism, angiogenesis, and tumor invasion [14]. Furthermore, the activation of AKT involves the phosphorylation of two regulatory phosphorylation sites, threonine-308 (Thr308) and serine-473 (Ser473) [17]. Co-phosphorylation of these two amino acids is essential for full activation of AKT [18]. Activated AKT specifically phosphorylates many downstream substrates harboring the recognition motif R-X-R-X-X-S/T-B (where X is any amino acid and B represents a bulky hydrophobic amino acid) in the cytosol and nucleus [16, 19-20] (Figure 1).

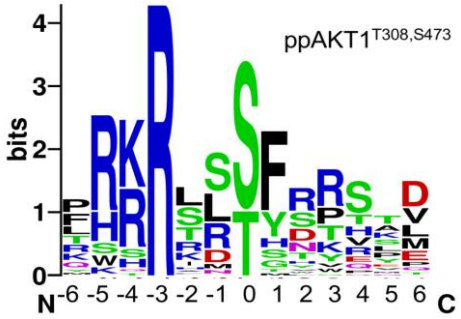

Figure 1
Target sequences of substrate recognition motif for Activated AKT (phosphorylated on S473 and T308) [20]

Various factors such as proteins, ligands, receptors and effector molecules play a role in AKT activation [21]. In this context, AKT serves as a central node within a signaling pathway that includes numerous components, and various cellular signaling pathways intersect at the AKT molecule. [14]. Therefore, AKT has a significant number and variety of downstream substrates or targets [22] (Table 1). Studies investigating these substrates are crucial because they provide an effective approach to discovering new therapeutic targets. In this study, we aimed to determine the potential AKT substrates in the presence of different protein kinase inhibitors in MDA-MB-231 breast adenocarcinoma cells which belong to the TNBC category. For this purpose, PKA, PI3K/PKB and PKCs, were targeted and an inhibitor of one of these proteins was used each time. Phospho-forms of AKT substrates were determined by Western blot analysis from cell lysates after stimulation with epidermal growth factor (EGF), which is one of the important factors that enable AKT activation.

Table 1
List of some of the known AKT substrates and their phosphorylation sites

GENE SYMBOL	GENE NAME	P-SITE	FUNCTION
FOXO1A	*Forkhead box O1A*	T24	FoxO Signaling
FOXO1A	*Forkhead box O1A*	S256	FoxO Signaling
FOXO1A	*Forkhead box O1A*	S319	FoxO Signaling
FOXO3	*Forkhead box O3*	S253	FoxO Signaling
FOXO3	*Forkhead box O3*	T32	FoxO Signaling
FOXO4	*Forkhead box O4*	S197	FoxO Signaling
FOXO4	*Forkhead box O4*	T32	FoxO Signaling
FOXO4	*Forkhead box O4*	S262	FoxO Signaling
NOS3	*Nitric oxide synthase 3*	S1177	NO Signaling
BRF1	*B - related factor 1*	S92	RNA Metabolism
PFKFB2	*6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2*	S466	Insulin and carbohydrate metabolism
PFKFB2	*6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2*	S483	Insulin and carbohydrate metabolism
CLK2	*CDC-like kinase 2*	S34	Insulin and carbohydrate metabolism
CLK2	*CDC-like kinase 2*	T127	Insulin and carbohydrate metabolism
BRCA1	*Breast cancer gene 1*	T509	DNA repair, modification, and chromatin regulation
BRCA1	*Breast cancer gene 1*	S694	DNA repair, modification, and chromatin regulation
ESR1	*Estrogen receptor 1*	S167	Hormone receptors
ESR1	*Estrogen receptor 1*	S305	Hormone receptors
AR	*Androgen receptor*	S791	Hormone receptors
AR	*Androgen receptor*	S213	Hormone receptors
AKT1	*RAC-alpha serine/threonine-protein kinase*	S246	Cell growth and survival
AKT1	*RAC-alpha serine/threonine-protein kinase*	T72	Cell growth and survival
BRAF	*B-Raf proto-oncogene, serine/threonine kinase*	S365	Cell growth and survival
BRAF	*B-Raf proto-oncogene, serine/threonine kinase*	S429	Cell growth and survival
TSC2	*Tuberous sclerosis complex 2*	S939	Cell growth and survival
TSC2	*Tuberous sclerosis complex 2*	S981	Cell growth and survival
MDM2	*Mouse double minute 2 homolog*	S186	Apoptosis
MDM2	*Mouse double minute 2 homolog*	S188	Apoptosis
MDM2	*Mouse double minute 2 homolog*	S166	Apoptosis
PDCD4	*Programmed cell death 4*	S67	Apoptosis
PDCD4	*Programmed cell death 4*	S457	Apoptosis

MATERIALS AND METHODS

Cell Culture

The MDA-MB-231 (ATCC, HTB-26) breast adenocarcinoma cells were obtained from American Type Culture Collection. The cells were incubated in an incubator (ESCO-CCL-170B-8) 5% CO_2 atmosphere, 90% humidified air at 37°C. The culture medium was DMEM high glucose medium (D5796, Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (F9665, Merck, Darmstadt, Germany) and 1% penicillin-streptomycin (15140122, Gibco, ThermoFisher Sci., Waltham, Massachusetts, USA) in 25 cm^2 culture flask (CLS430639, Corning, New York, USA). Cell morphologies and confluency were visualized using an inverted microscope (Primovert, Zeiss, Jena, Germany).

EGF Stimulation and Treatment of Cells with PKA, PI3K/PKB and PKC Inhibitors

The MDA-MB-231 cells were seeded into a 6-well culture plate at 1×10^5 cells/well concentration. The day before treatment, the cells were incubated in serum-free media overnight and then treated with protein kinase inhibitors: H-89 Dihydrochloride (MedChemExpress, HY-15979) as a PKA inhibitor, LY294002 (Thermo Fisher Scientific, Inc., PHZ1144) as a PI3K/PKB inhibitor, and RO318220 (Abcam, ab120374) as a PKC inhibitor. After washing the cells with 1×PBS, they were treated for 2 hours with two different inhibitor concentrations: 2 and 5 μM for H-89 Dihydrochloride (PKA inhibitor) [23], 1 and 5 μM for LY294002 (PI3K/PKB inhibitor) [24], and 1 and 5 μM for RO318220 (PKC inhibitor) [25]. Following treatment, the cells were stimulated with EGF (10 ng/mL) in medium containing 1% FBS and incubated for 72 hours [26]. Cells that were not treated with EGF or inhibitors were used as controls.

Western blot

Following treatment with inhibitors and EGF described above at the indicated times, the cells were harvested and the cell lysates were prepared from the untreated and treated MDA-MB-231 cells. Cell lysates were obtained by 1X cell lysis buffer (cat.9806, Cell Signaling Tech., USA) and incubated on ice for 30 min. The cell lysate samples were clarified by centrifugation at 10.000 rpm for 1 min, and the total protein concentration was determined by the Bradford method. A hundred micrograms of total protein were size-fractioned on a 10 % SDS page and transferred to the PVDF membrane (cat. 88518, ThermoFisher Sci., USA). For immunoblotting, the membranes were incubated overnight with primary antibody (1:1000) (rabbit Phospho-Akt Substrate (cat. 9614, Cell Signaling Tech., USA) and then with horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. sc-2357, Santa Cruz Biotechnology, Inc., USA) (1:5000) for 2 h. The protein immunoreactivity was visualized using X-ray imaging.

Statistical Analysis

Analyses of experimental data were performed using GraphPad Prism 9.0 software. *p* values of the results were calculated using One-way ANOVA (Dunnet t-test). A *p* value of <0.05 was considered statistically significant. The results were normalized and presented by determining ± standard error values.

RESULTS

To determine the potential AKT substrates in the presence of different protein kinase inhibitors, the MDA-MB-231 cells were treated or untreated with H-89 (PKA inhibitor), LY294002 (PKB inhibitor) or RO318220 (PKC inhibitor) and then subsequently exposed to EGF or not. Whole-cell lysates were blotted with phospho-AKT substrate antibody. In this study, we demonstrated that the phosphorylation level of an approximately 30 kDa AKT substrate (pp30), which has not been previously

described in the literature, varies depending on the presence of EGF and the specific inhibitors. We found that the phosphorylation level of pp30 increased by 1.11 to 1.44-fold with EGF stimulation compared to the control. In the presence of inhibitors, the phosphorylation level of pp30 decreased in a dose-dependent manner in both control and treated cells. Despite PKA and PKB inhibition, phosphorylation still increased with EGF, although PKC treatment suppressed this increase by %52 at 5 μM concentration. These results indicate that AKT targets are regulated not only by the PI3K/AKT axis but also by the PKC pathway under EGF stimulation. In this context, it can be inferred that AKT, which is known to have an inverse relationship with PKC signaling in cancer cells, could interacts with some of AKT substrates (Figure 2).

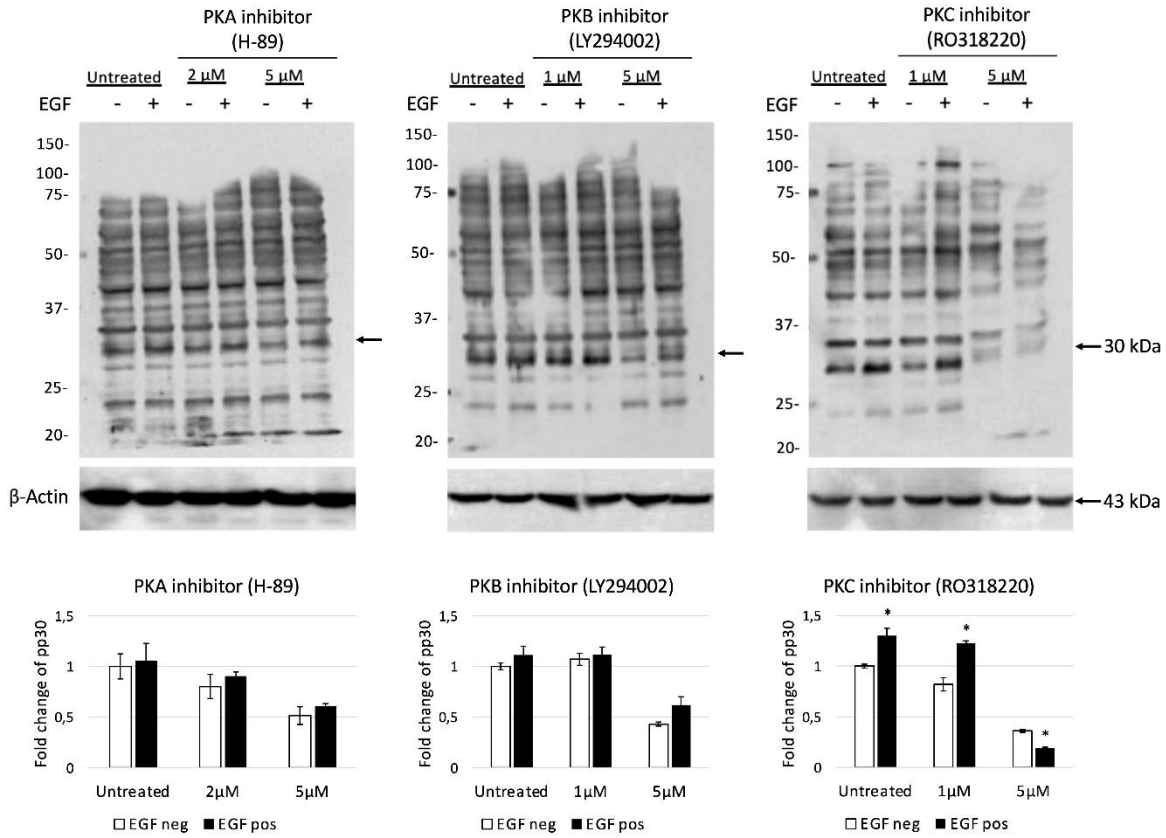

Figure 2

Western blot analysis of various phosphorylated AKT substrates in MDA-MB-231 cells, treated or untreated with PKA, PI3K/PKB, and PKC ınhibitors upon EGF stimulation, revealed a 30 kDa Phospho-protein (pp30) (black arrows) (, p<0.05).*

DISCUSSION

AKT (PKB) was discovered about 32 years ago and has since been the subject of tens of thousands of studies in various fields of medicine and biology [27]. The broad range of AKT's functions arises from its numerous downstream substrates, with over 200 AKT substrates identified to date [27-28]. The molecular interactions of the AKT signaling network are better understood by determining and identifying these diverse AKT substrates [27]. This study presents a method for identifying novel AKT substrates using specific inhibitors and phosphomotif-specific antibodies. The method involves activating the kinase through stimulation and comparing phosphoproteins based on the presence or absence of pathway blockade [29]. Using this approach, this study determined a novel candidate AKT substrate (pp30) regulated also by PKC (Figure 2). Similarly, Kane et al. [29] applied this method and isolated a novel 160-kDa substrate for AKT from adipocytes. Then, the characterization studies of the

protein revealed that the 160 kDa protein was a GTPase activating protein for Rab. AKT-mediated phosphorylation of this protein plays an important role in insulin-stimulated transport of the glucose transporter GLUT4 to the plasma membrane [30]. Another giving evidence, four novel phospho-proteins (47, 75, 105, and 250 kDa) were identified using an AKT phosphomotif-specific antibody through insulin stimulation [31]. In addition to this, the lack of change in the phosphorylation level of the described 75 kDa protein upon treatment with PI3K inhibitors suggests that this protein may not be an AKT substrate [31]. According to these findings, it can be said that the combination of different inhibition strategies is needed to confirm the data obtained.

The relationship between PKC inhibitor RO318220 and PI3K/AKT signal pathway was examined by Zhu et al [32]. They showed that adding the PKC inhibitor RO318220 increased AKT phosphorylation and neuronal survival suggesting that RO318220 protects rat cerebellar granule cell neurons by activating AKT [32]. Similarly, Wen et. al [33] showed that PKC inhibitors RO318220, bisindolylmaleimide VIII, and PKCβ inhibitor LY379196 increased phosphorylation and stimulation of AKT in human airway epithelial A549 cells and human embryonic kidney HEK293 cells. In another study, the regulation and role of mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling in uveal melanoma responses to PKC inhibition were investigated [34]. They indicated that in UM patients treated with the PKC inhibitor IDE196, the PI3K/AKT pathway was active and not inhibited by PKC inhibition, whereas the (MAPK) signaling pathway was inhibited [34].

The results of the present study are preliminary and demonstrate how new AKT targets can be identified through the cooperation of different pathways. Whether pp30 is indeed phosphorylated by AKT can be confirmed through immunoprecipitation and *in vitro* kinase assays. Additionally, proteomic approaches using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight/Time-of-Flight Mass Spectrometry (MALDI-TOF/TOF-MS) are needed for molecular characterization. In the next stage, it will be important to determine the phosphorylation site and cellular function of pp30.

In conclusion, studies investigating novel AKT targets are crucial for identifying new therapeutic options. Moreover, the methodology used in this study demonstrated that cellular pathways can crosstalk to achieve common goals. Different enzymes, such as PKC, can activate distinct signaling pathways and targets, including AKT substrates.

Ethical Statement

This study is an original research article designed and developed by the authors.

Ethics Committee Approval

This study does not require any ethics committee approval.

Author Contributions

Research Design (CRediT 1) M.O.T. (%30) - Ş.P. (%30) – S.P. (%40)
Data Collection (CRediT 2) M.O.T. (%25) - Ş.P. (%25) – S.P. (%50)
Research - Data Analysis – Validation (CRediT 3-4-6-11) M.O.T. (%30) - Ş.P. (%30) – S.P. (%40)
Writing the Article (CRediT 12-13) M.O.T. (%40) - Ş.P. (%30) – S.P. (%30)
Revision and Improvement of the Text (CRediT 14) M.O.T. (%40) - Ş.P. (%20) – S.P. (%40)

Financing

This research was not supported by any public, commercial, or non-profit organization.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals (SDG)

Sustainable Development Goals: 3 Healthy and quality life

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