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Role of Raf Kinase Inhibitor Protein in Pathophysiology of Prostate Cancer

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Abstract

Raf kinase inhibitor protein (RKIP) is a small, cytosolic protein named for its ability to block Raf-mediated activation of MAPK and ERK. It also block G-protein signaling and NF-κB activation. An in vitro screen to identify genes that regulate prostate cancer (PCa) metastasis revealed that expression of RKIP was decreased in high versus low metastatic PCa cells. Modulation of RKIP expression revealed that it inhibited invasion and loss of RKIP promoted in vitro invasion. Animal studies were used to demonstrate that RKIP could inhibit PCa metastasis from orthotopically injected tumor cells without an effect on primary tumor growth. Taken together, these results indicated RKIP acted as a PCa metastasis suppressor gene. Evaluation of RKIP expression in clinical cases of PCa revealed that RKIP expression was moderate to high in non-neoplastic prostate, low in 50% of primary prostate cancers, and absent to low in the majority of metastases. Furthermore, low RKIP expression in primary prostate tumors was predictive of early tumor recurrence. Loss of RKIP was shown to induce resistance to radiation in PCa cells in vitro and in an in vivo murine model. Taken together, these studies indicate that RKIP plays multiple roles in PCa pathophysiology, suggesting that a method to increase RKIP expression in PCa may have therapeutic benefits.

Keywords

protein kinase; metastasis; ERK; MAP kinase

I. RAF KINASE INHIBITOR PROTEIN

Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, is a conserved, small, cytosolic protein originally purified from bovine brain.[1] RKIP (also known as PEBP-1 and PBP) has wide tissue expression in a variety of different mammalian species such as monkey,[2] rat,[3] chicken,[4] and human.[5,6] The PEBP family of proteins is highly conserved and does not share significant homology with any other protein family.[7] Human RKIP mRNA is 1434 bp long and is transcribed from a gene composed of 4 exons spread across approximately 10 kb[5] of chromosome 12q24.23 with a *PEBP* homologue on chromosome 2p.[8,9] Human RKIP mRNA shares a 95% similarity to bovine mRNA and an 85.5% similarity to rat mRNA.[6] Human RKIP mRNA encodes a 187 amino acid protein that shares a 186 amino acid overlap with the bovine 21 kDa RKIP and a 187 amino acid overlap with rat 23 kDa RKIP.[5]

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II. RKIP IN SIGNALING

II.A. RKIP in the Raf Signaling Pathway

RKIP has been shown to be involved with several cell signaling cascades. A yeast two-hybrid assay screen of a human T cell library was used to identify proteins that bound to Raf-1 kinase binding domains.[10] The protein identified was designated RKIP, whose sequence was analogous to the human and monkey 23 kDa protein PEBP.[10] Using anti-RKIP antibody, antisense RKIP and sense RKIP expression vectors, Yeung et al. discovered that RKIP could bind Raf-1 and MEK-1, and weakly bind to ERK-2, interfering with MEK phosphorylation and activation by Raf-1. However, RKIP was not a substrate for Raf-1 or MEK. RKIP did not bind to Ras, nor possess kinase activity. It appears that RKIP acts to set the threshold for Raf-1 activation and subsequent activation of the MEK/ERK pathway. Raf-1 dissociates from its complex with MEK in the presence of RKIP. As a result, downstream mitogen activated protein kinase (MAPK) signaling is interrupted and diminished. As stated earlier, RKIP can bind to Raf-1 or MEK, yet not at the same time, and binding to either one is enough to cause downstream inhibition.[11] In addition, it was postulated that RKIP may be involved in growth, transformation, and differentiation,[10] which are often deregulated in many forms of cancer.

Evidence for a role for RKIP in cell growth comes from its interaction with protein kinase C (PKC), which phosphorylates proteins that regulate growth, differentiation, and transcription. PKC phosphorylates RKIP on serine 153 and alleviates RKIP's inhibition of Raf-1.[12] PKC is normally recruited to the plasma membrane and activated by diacylglycerol, whose location near the plasma membrane may place it in close proximity to RKIP, which also binds to phospholipids present in cell membranes.[13]

II.B. RKIP in NF-κB Signaling

Expression of RKIP altered NF-κB signaling induced by tumor necrosis factor alpha (TNF-alpha), independent of its regulatory effects upon the MAPK cascade.[14] This regulation occurred upstream of NF-κB as a result of inhibition of IKKβ. As a result, IKK can no longer phosphorylate or activate IκB, allowing for NF-κB to remain sequestered with IκB. Interacting in a similar fashion with Raf-1 and MEK, RKIP was found to inactivate several kinases of the NF-κB family.[14] RKIP appears to have multifunctional roles concerning regulation of different cell signaling cascades.

II.C. RKIP in G Protein Signaling

G protein signaling has been shown to be facilitated by RKIP.[15] G protein–coupled receptor kinase 2 (GRK-2) is a critical negative feedback inhibitor of G protein–coupled receptors (GPCR). GRK-2 phosphorylates activated GPCRs, which uncouples them from the active G protein GPCR complex resulting in initiating GPCR internalization and recycling and inactivating G protein signaling. The importance of GRK-2 activity in controlling G protein signaling implies that GRK-2 activity needs to be tightly regulated. It was demonstrated that RKIP is a physiological inhibitor of GRK-2.[16] Specifically, after stimulation of GPCR, RKIP dissociates from Raf-1 and associates with GRK-2 and blocks its activity. This switch is triggered by the protein kinase C (PKC)–dependent phosphorylation of the RKIP on serine 153.[12,16]

II.D. Signaling Summary

In summary, RKIP impacts several signaling pathways and intriguingly demonstrates a new paradigm in signal transduction. Namely, a receptor that activates PKC may promote activation of several different signaling pathways through both inducing release of RKIP

from Raf, promoting MEK/ERK signaling, and inducing association of RKIP to GRK-2, which promotes G protein signaling.

III. IDENTIFYING RKIP AS A METASTASIS SUPPRESSOR GENE

In order to determine what genes may regulate metastasis, we wanted to compare gene expression in low versus high metastatic cell lines. To that end, we use the LNCaP prostate cancer cell lines, which have a very low metastatic rate, and its derivative cell line C4-2B that have a high metastatic rate. To derive C4-2B, LNCaP cells were injected into the mice and rare bone metastatic tumors were obtained, made into single-cell suspension, and reinjected into mice. This process was repeated several times until the highly metastatic C4-2B cell line was derived. Since these two cell lines were very similar, with a key difference being metastatic rate, we used them to identify genes whose expression was altered in the highly metastatic phenotype compared to the low metastatic phenotype. Using gene array, we found that C4-2B prostate cancer cells had much less RKIP expression than nonmetastatic prostate cancer cells.[8] This was further confirmed in a small sample of prostate cancer tissues from patients in which RKIP expression was downregulated or undetectable in prostate cancer metastases. This result provided evidence that RKIP could be clinically relevant.

To determine if RKIP had a functional role in metastasis and the metastatic phenotype, we first evaluated the role of RKIP expression on in vitro invasion.[8] We knocked down RKIP expression in LNCaP cells, which resulted in increasing their invasive ability. Additionally, restoring RKIP expression in the C4-2B cell line resulted in decreasing their invasive ability. These changes in RKIP expression were associated with corresponding and expected changes in ERK expression. Specifically, basal ERK phosphorylation was higher in C4-2B cells than LNCaP cells, and increasing RKIP expression reduced ERK phosphorylation; whereas, decreasing RKIP expression increased ERK phosphorylation. Thus, taken together, loss of RKIP in prostate cancer cells promotes invasion and dysregulated ERK activation.

We next wanted to determine if RKIP modulated the prostate cancer metastasis in an in vivo model. To perform this, C4-2B cells were stably transfected with RKIP to restore RKIP expression. Then control transfected or RKIP transfected cells were injected orthotopically into the mouse prostate, and lung metastases were quantified. We found that RKIP restoration in the highly metastatic C4-2B prostate cancer cells reduced spontaneous lung metastasis, but not primary tumor growth rate. These observations were consistent with RKIP being a metastasis suppressor gene. Since this original finding in prostate cancer, there has been increasing evidence that RKIP plays a role as a metastasis suppressor gene in multiple other cancers, as highlighted in other aspects of this special issue.

The mechanisms through which RKIP suppresses metastasis are not known. However, increased RKIP expression was associated with decreased vascular invasion and decreased ability to form new blood vessels in the primary tumors in the rodent model,[8] suggesting that loss of RKIP may act at the early angioinvasive stages of metastasis. Additionally, the use of a proteasome inhibitor, NPI-0052, was shown to inhibit epithelial mesenchymal transition (EMT) through induction of RKIP expression,[17] suggesting that loss of RKIP could lead to EMT, which is considered to promote metastasis. The mechanism through which RKIP expression is decreased during prostate cancer progression is unclear, but there is evidence that Snail represses RKIP transcription.[18] Taken together, these data demonstrate that RKIP is a clinically relevant prostate cancer metastasis suppressor gene that regulates the Raf/MEK/ERK pathway. RKIP could be a promising molecular target for compounds designed for cancer treatment.

IV. RKIP AS A RADIOSENSITIZER

Radiotherapy is often used as a curative treatment for localized prostate cancer. However, approximately 30% of cases reoccur depending on the stage of the tumor. Additionally, radiotherapy can be used as a palliative therapy for widespread disease. Thus, improved efficiency of radiotherapy may improve therapeutics for prostate cancer. RKIP has been previously shown to promote chemotherapy-induced apoptosis. We explored if this property extended to radiotherapy-induced apoptosis. We found that ionizing radiation induced RKIP expression.[19] Knockdown of RKIP in prostate cancer cells, which would be similar to what is found in advanced prostate cancer, resulted in increasing resistance to radiotherapy. In contrast, reexpressing RKIP in the C4-2B cells that have low endogenous RKIP expression resulted in enhancing their sensitivity to radiation in vitro. In correlation with this, we found that PARP cleavage, an indicator of apoptosis, was increased in cells overexpressing RKIP and decreased in cells in which RKIP was knocked down.

In order to determine if these in vitro studies extended to in vivo effects, mice were injected with tumor cells in which RKIP expression was modulated, and then subjected to radiotherapy of 2Gy for 15 cycles, similar to a clinical radiotherapy protocol. We observed that knockdown of RKIP protected the tumors from radiotherapy.[19] Additionally, it was found that knockdown of RKIP was associated with decreased expression of several apoptosis-related genes including PDLIM5, ID2, DAPK1, and ATRX. Taken together, these results indicate that loss of RKIP, as is observed in advanced prostate cancer, induces radioresistance in tumors. This observation suggests that increasing RKIP expression may radiosensitize tumors and enhance therapy of prostate cancer.

V. RKIP EXPRESSION IN CLINICAL PROSTATE CANCER

In order to determine if RKIP has any prognostic value in clinical prostate cancer, we examined several tissue microarrays for RKIP expression. The tissue microarrays encompassed a spectrum from non-neoplastic prostate tissue, primary tumors, and metastatic tumors. We identified that RKIP expression was strong in the majority of non-neoplastic tissues, was low in approximately 50% of primary tumors that are Gleason score 6 and 7 and strong in the remaining 50%, and was low in the majority of metastatic tissues.[20] These findings are consistent with RKIP being a metastasis suppresser gene.

The observation that RKIP was low in 50% of primary tumors suggests that its expression could serve as a prognostic factor. Accordingly, we evaluated for the ability of RKIP to predict tumor recurrence based on a rise in serum prostate specific antigen (PSA) levels. PSA is a serum biomarker that reflects the amount of tumor burden in prostate cancer. After initial therapy, PSA levels decline, and a rise in PSA levels post-therapy indicates tumor recurrence. We found that low RKIP expression in primary tumors predicted a lower five-year recurrence-free survival (61%) compared to those that had high RKIP (88%).[20]

VI. TARGETING THE RKIP PATHWAY

RKIP presents a challenge in terms of therapeutic targeting. Specifically, in the case of metastasis, RKIP expression is decreased in cancer cells. Thus, to reverse the phenotype associated with decreased RKIP, one could consider increasing RKIP in cancer cells, for example, through gene therapy. However, this is challenging because it may be necessary to target all cancer cells because even one cell with low RKIP has the potential to become metastatic. Unfortunately, there currently are no therapies that can efficiently replace specific gene expression in all cancer cells in vivo. Several alternative options exist. One potential method is to identify compounds that induce RKIP expression. The ability to chemically induce RKIP expression has the potential to impact all cells. In addition to the

decreased metastatic potential, inducing RKIP expression may sensitize cells to chemotherapy-induced apoptosis.[21] An alternative strategy to consider would be to target pathways that are increased due to diminished RKIP expression. For example, decreased expression of RKIP induces MEK, NF–κB, and G protein activation; thus, targeting these signaling pathways has the potential to diminish the prometastatic activity associated with decreased RKIP expression. Currently, it is not clear how these different pathways interact to confer the metastatic phenotype and which of these pathways is the most important to target. Further exploration of the biology of RKIP and its role in the pathogenesis of cancer progression and metastasis is necessary to define potential therapeutic agents that can take advantage of RKIP's role in metastasis to impact the development of prostate cancer metastasis.

VII. CONCLUSIONS

Although the existence of RKIP, initially identified as PEBP, has been recognized for many years, only recently has its role in cell signaling been truly appreciated. This has led to a recent flurry of activity to understand its roles in physiology and pathophysiology. Clearly, due to its role in modulating PKC signaling through regulation of Raf and G protein signaling, RKIP has the potential to modulate many processes. This is reflected in the myriad of functions it performs in different tissues. At this early stage of discovery regarding RKIP's role in many signaling pathways, it is too early to confidently reconcile the contribution of RKIP to different biological processes with its role in signaling. Furthermore, additional studies are needed to determine the precise relationship between RKIP and its role in several diseases, including Alzheimer's disease, diabetes, and cancer. The more that is defined about RKIP and its role in health and disease, the more it may help identify RKIP or proteins downstream of RKIP as important targets for therapeutic interventions.

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ABBREVIATIONS

ERK	extracellular-regulated kinase
GRK	G protein-coupled receptor kinase
GPCR	G protein-coupled receptor
MAPK	MAP kinase
MEK	MAP regulated/ERK regulated kinase
NF-κB	nuclear factor κB
PCa	prostate cancer
PEBP	phosphatidylethanolamine-binding protein
PKC	protein kinase C
PSA	prostate specific antigen
RKIP	Raf kinase inhibitor protein
TNF	tumor necrosis factor

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