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Phosphatidylethanolamine Binding Protein aka Raf Kinase Inhibitor Protein: A Brief History of Its Discovery and the Remarkable Diversity of Biological Functions

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Abstract

Phosphatidylethanolamine-binding protein (PEBP) was identified almost three decades ago as an abundant protein in bovine brain. PEBP is the prototype of a highly conserved family of proteins represented in all three major phylogenetic divisions, *eukaryota*, *bacteria*, and *archaea*, with no significant sequence homology to other proteins. PEBP proteins have been studied in many species. The most thoroughly explored biological role of PEBP is that of a modulator of intracellular signaling pathways, which is mediated by its ability to bind and inhibit a number of protein kinases. The first such interaction that came to light was with the Raf1 kinase, and PEBP is thus widely referred to in the literature under its alternate name RKIP (Raf kinase inhibitory protein). The activity of RKIP itself is subject to regulation by phosphorylation. Intriguingly, PEBP has also been reported to possess additional, and diverse, biological functions unrelated to protein kinase networks that remain to be investigated in detail. Recent findings that RKIP may function as a suppressor of cancer metastasis are of great interest and importance. Prognostic and therapeutic applications of RKIP in human cancer were the subject of the first international workshop on RKIP that was held at the University of California, Los Angeles, in March 2010. This paper was presented at the workshop as a summary of the history of this still small but rapidly evolving field.

Keywords

cancer; signal transduction; metastasis; tumor suppression; phosphorylation; apoptosis; MAP kinase pathway; NF- κ B pathway; G protein-coupled receptor pathway

I. DISCOVERY AND EARLY STUDIES

In 1984, Bernier and Jollés reported the purification of a basic 23 kDa cytosolic protein from bovine brain,¹ which they subsequently discovered displayed affinity for phosphatidylethanolamine without apparent catalytic activity.² In 1987, this group reported the complete amino acid sequence of the 186 residue protein³ and went on to speculate that it played a nonenzymatic role in the metabolism of lipophilic compounds.⁴ In completely independent studies, in 1983 Jones et al. discovered the same protein in detergent extracts of mature rat spermatozoa.⁵ Subsequent work from this group showed that the protein is found in epididymal secretions as well as sperm plasma membranes,⁶ and in 1991, partial sequencing revealed its identity with the 23 kDa protein identified by the group of Jollés.⁷ They speculated that the affinity of the protein for phospholipids could account for the organization of sperm plasma membranes into specific regional domains. Both of these lines of investigation were largely enabled by the abundance of the protein in the tissues under

investigation. Current annotation designates the gene that encodes this protein as *PEBP1*, although for a time it was listed in GenBank as *PBP* (for prostatic binding protein).

II. THE SECOND DECADE—REMARKABLE CONSERVATION AND DIVERSITY OF FUNCTION

In the 1990s, papers reporting on this interesting protein continued to accumulate, as did its putative biological functions. In 1990, Grandy et al. reported the purification of a 23 kDa protein from rat brain using morphine affinity columns,⁸ and Robinson and Tatchell identified the *S. cerevisiae TFS1* gene in a genetic screen for suppressors of *CDC25* mutations (*CDC25* is a guanine exchange factor for the yeast homologs of the RAS oncoproteins).⁹ In both cases, molecular cloning and sequencing of the cDNA combined with the advent of public sequence databases led to a rapid association with the 23 kDa protein of Jollés and Jones. Although Grandy et al. concluded that PEBP was unlikely to be the opioid receptor they were seeking, in 1994 PEBP was reported as a putative *Drosophila* odorant-binding protein expressed in olfactory hairs,¹⁰ and recent work has implicated PEBP in binding of the endogenous opioid morphine-6-glucuronide in plasma.^{11,12} Little work has been done on yeast *TFS1* until recently, when it was shown to directly interact with and inhibit the IRA2 GTPase-activating protein.^{13–15}

In 1992, the group of Ojika reported the purification from rat brain of a 11 amino acid peptide capable of stimulating acetylcholine synthesis in specific regions of the hippocampus, which they designated HCNP (for hippocampal cholinergic neurostimulating peptide).¹⁶ However, it was not until 1995 when the cDNA was cloned that it was revealed that HCNP corresponds precisely to the 11 N-terminal residues of PEBP1.¹⁷ Also, in 1995 a PEBP ortholog was identified in the parasitic nematode *Toxocara canis* as an abundant secreted protein with an implicated role in immune evasion¹⁸ (and in 1996 in another parasitic nematode, *Onchocerca volvulus*¹⁹). In 1996, Bradley et al. reported the regulation of *Antirrhinum* (snapdragon) flower position and morphology by the *CEN* gene,²⁰ whose cloning and sequencing revealed strong similarity to PEBP. PEBP orthologs with roles in flowering and meristem identity were subsequently reported in *Arabidopsis*²¹ and *Solanum* (tomato).²² To bring this amazing parade of biological functions and, hence, implied biochemical activities to a close, in 1998 Bruun et al. reported that a high affinity inhibitor of yeast carboxypeptidase Y designated I_c is encoded by the *TFS1* gene.²³

III. THE THIRD DECADE—MODULATION OF INTRACELLULAR SIGNALING NETWORKS

In 1999, the groups of Kolch and Sedivy reported that PEBP1 binds to the RAF1 protein kinase, inhibits its activity, and suppresses signaling through the mitogen-activated protein kinase (MAPK) pathway.²⁴ In that paper, these groups coined the name RKIP (for RAF1 kinase inhibitor protein), which has become a widely used designation for the protein in papers focusing on its role in mammalian intracellular signaling. RKIP was in fact discovered over five years earlier by these groups in a yeast two-hybrid screen for interactors of the RAF1 kinase domain (this paper does not mention PEBP by name and focuses instead on the 14-3-3 protein, which was the most abundant hit in the screen).²⁵ Although RKIP was a single hit in the original screen, the interaction with RAF1 was strong. This stimulated further experiments, taking four years of concentrated effort in the two labs to elaborate the molecular mechanism of its action in the MAPK pathway. This discovery provided an important precedent and framework for the role of PEBP as a regulator of intracellular kinase signaling pathways and stimulated a burst of activity in a number of laboratories to understand the underlying mechanisms in this important area.

III. A. MAPK Pathway

MAPK signaling pathways are highly conserved and involved in cell growth, differentiation, survival, and invasion. They constitute regulatory modules composed of three kinases that act in succession: a MAP kinase kinase kinase (MAPKKK), which phosphorylates a MAP kinase kinase (MAPKK), which in turn phosphorylates a MAP kinase [(MAPK), Fig. 1]. There are three major pathways, each named after the terminal MAPK: the extracellular-signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK or SAPK1) pathway, and the p38 MAPK (SAPK2/RK) pathway. In general, ERK1/2 are the key transducers of proliferation signals and are often activated by mitogens. In contrast, JNK and p38 MAPK are poorly stimulated by mitogens but strongly activated by a variety of cellular stresses.

Many different growth factors that signal through transmembrane tyrosine kinase receptors activate the ERK/MAPK pathway through the small G protein RAS, which consequently binds RAF1 kinase and thereby recruits RAF1 to the inner surface of the cell membrane.²⁶ RAF1 thus acts as the MAPKKK in the ERK/MAPK pathway. Once localized to the membrane, RAF1 becomes activated and phosphorylates and activates the MAPKK MEK, which in turn phosphorylates and activates ERK. Phosphorylated ERK translocates into the nucleus and regulates gene expression via interaction with various transcription factors, such as CREB, AP-1, ETS, and MYC.

The events that lead to RAF1 activation following membrane recruitment are still incompletely understood, but almost certainly involve multiple phosphorylations and dephosphorylations.^{27,28} These are believed to result in conformational changes that lead to the exposure of the RAF1 N-terminal regulatory domain. RAF1 has been purified as part of a yet poorly defined 300–500 kDa complex composed of possibly more than 30 interacting proteins.²⁹ Examples of RAF1 interactors include the 14-3-3 adapter proteins; HSP90 chaperone; PP2A, and CDC25 phosphatases; AKT, PKC, JAK, KSR, MEK, and ERK kinases; GRB adaptors, and RKIP. This elaborate network of RAF-interacting proteins is believed to be a key regulatory element of the entire MAPK signaling cascade.

RKIP has emerged as a main inhibitory component of this network (Fig. 1). RKIP directly interacts with both RAF1 and MEK, and disrupts the RAF1/MEK interaction, thereby preventing the activation of MEK and downstream components of the signaling cascade. The activity of RKIP is in turn regulated by phosphorylation by protein kinase C (PKC), which inhibits its interaction with RAF1 and hence antagonizes its inhibition of MAPK pathway signaling.^{30,31} RKIP has also been reported to interact with the BRAF isoform, although its effects on BRAF activity have not been consistent.^{32,33}

An interesting recent observation links RKIP, the MAPK pathway, and the spindle assembly checkpoint in mitosis.³⁴ Both PKC-phosphorylated RKIP and activated RAF1 were localized to early mitotic kinetochores, and depletion of RKIP during M phase led to bypass of the spindle assembly checkpoint and generation of chromosomal abnormalities. Depletion of RKIP caused inhibition of Aurora B kinase activity and decreased its localization to kinetochores, and inhibition of MAPK pathway activity rescued the effect of RKIP loss. The phosphorylation substrate(s) of the MAPK pathway in mitosis is(are) not Aurora B and has(have) not been identified, but may be component(s) of the kinetochore “passenger protein complex,” which consists of, in addition to Aurora B, INCENP, Survivin, and Borealin/Dasra B.³⁵

III. B. NF- κ B Pathway

The action of RKIP is however not limited to a single signaling cascade. In the NF- κ B signaling pathway (Fig. 1), RKIP has been shown to interact with the activating kinases I κ B

kinase α (IKK α), I κ B kinase β (IKK β), transforming growth factor β -activated kinase (TAK), and NF- κ B-inducing kinase (NIK).³⁶ It is interesting to note that these kinases, like RAF1, belong to the MAPKKK superfamily. However, while RKIP can clearly interact with several MAPKKKs, the interactions are specific since other MAPKKKs tested show no detectable interaction. Release of RKIP from TAK, IKKs, and NIK, by an as yet incompletely understood mechanisms,³⁷ results in the phosphorylation and degradation of I κ B, which in turn releases the NF- κ B heterodimer that subsequently translocates into the nucleus and initiates transcription from its target promoters. More recent work suggests that RKIP may play a role of a scaffold protein in the NF- κ B pathway,³⁷ and PEBP4 (the second PEBP family member encoded in the human genome) has been reported to act as a scaffold in the MAPK pathway.³⁸ In a biological context, it has been shown that ablation of RKIP enhances NF- κ B-mediated transcription, whereas overexpression of RKIP reduces it.³⁶ Because NF- κ B can promote survival by antagonizing apoptotic processes, loss of RKIP would be expected to lead to increased NF- κ B signaling and decreased susceptibility to apoptosis; this has indeed been demonstrated in an in vitro prostate carcinoma model.³⁹

III. C. GPCR Pathway

The third important area of RKIP action is in G protein-coupled receptor (GPCR) signaling (Fig. 1). This was revealed by the discovery that RKIP can bind and inhibit the activity of the GPCR kinase GRK2.^{40,41} GRK2 acts to phosphorylate GPCRs, which uncouples the receptors from their associated G-proteins and interrupts the signaling pathway. Thus, inhibition of GRK2 activity by RKIP promotes GPCR signaling. It is of considerable interest that while phosphorylation of RKIP by PKC inhibits its interaction with RAF1, it promotes the interaction with GRK2. Thus, activation of PKC, resulting in the phosphorylation of RKIP, has the dual effect of activating both the MAPK and GPCR pathways: the former by relieving the inhibition of an activating kinase (RAF1) and the latter by imposing inhibition on an inhibitory kinase (GRK2).

IV. STRUCTURAL STUDIES

The first structures of human and bovine PEBP were reported independently in 1998 by the groups of Brady⁴² and Serre.⁴³ With few exceptions, PEBP proteins from diverse species have been found to be relatively easy to express and crystallize, leading to the publication of numerous structures, including those from plants, bacteria, yeast, and the protozoal parasite *Plasmodium*.^{44–47} The small size of the proteins has also enabled recent NMR structural studies.^{48–50}

Structural studies have had an important influence on the field. First, it soon became apparent that PEBP proteins possess a compact and stable structure based on a unique fold of a large, central β -sheet formed from five antiparallel strands. Second, PEBP proteins contain a characteristic shallow binding pocket terminating in a narrow cleft. This information was critical for the design of mutations in surrounding residues, which revealed that the binding pocket is necessary for the activity and function of the protein.

Early structures contained arsenate or phosphate ligands in the deep cleft of the pocket,^{42,43} which is also the region of highest structural conservation among different PEBP proteins. The realization that PEBP participates in diverse phosphorylation-dependent protein-protein interactions led to suggestions that the pocket may be a docking site for phosphorylated residues on partner proteins, and some recent data are in agreement with this interpretation.⁵¹ Other recent data have implicated phosphatidylethanolamine (and other related phospholipids) as ligands, which do not place a phosphoryl group in the cleft, and instead, point to the existence of a flexible pocket that relies on hydrophobic interactions for ligand binding.^{48,52} The identity of the physiological ligand(s) remains under debate to this

day and is discussed in more detail by Eves and Rosner, as well as Simister et al. (this volume).

IV. A. Phylogenetic Analysis

Structural studies combined with phylogenetic analyses of sequence databases have revealed that PEBPs comprise a remarkably conserved family represented in all three major phylogenetic divisions: archaea, bacteria, and eukaryota. The number of genes found in a single species ranges from two (human, mouse) to >20 (maize, rice),⁵³ but is typically in the lower range (for example, seven genes have been documented in *Drosophila*).⁵⁴ Given its relatively high expression, mammalian genomes contain numerous processed pseudogenes.⁵⁵ This complicates the determination of functional genes by *in silico* methods; for example, the mouse genome contains an intronless *PEBP* gene that presumably arose as a processed pseudogene and has been shown to be expressed in a testis-specific manner (designated as *Pebp2*).⁵⁶ No *PEBP2* ortholog exists in the human genome, and both mouse and human genomes contain additional non-expressed pseudogenes located at nonsynthetic positions, pointing to a relatively recent origin by retrotransposition.⁵⁵

V. ROLE OF RKIP IN CANCER

It is well documented that aberrant regulation of the signaling pathways under consideration here can promote the development of cancer. It is thus not surprising that RKIP has received considerable scrutiny in this context. A number of laboratories have played key roles in these efforts: the group of Kolch in MAPK signaling, the group of Yeung in NF- κ B signaling, the group of Rosner in mitotic checkpoints, and the groups of Keller and Rosner in metastatic processes. This volume is dedicated to the role of RKIP in cancer and contains many important updates in our efforts to understand these mechanisms.

Early investigations were quite fruitful and revealed that RKIP expression is reduced in several common cancers: prostate,⁵⁷ breast,⁵⁸ melanoma,³² colorectal carcinoma,⁵⁹ hepatocellular carcinoma,⁶⁰ and insulinoma.⁶¹ A number of recent reports have confirmed and expanded the range of cancers that frequently display reduction (or loss) of RKIP expression.^{62,63} On the basis of this general expression pattern, RKIP can be classified as a tumor suppressor. Resistance to chemotherapy is one implicated mechanism: treatment of several breast and prostate cancer cell lines with chemotherapeutic agents induced RKIP expression and predisposed the cells to apoptotic death by downregulating NF- κ B and RAF1 signaling.³⁹ Evolution of chemoresistance was in some cases correlated with a reduction of RKIP expression, and reexpression of RKIP was able to resensitize the cells.

An important turning point for the RKIP field was the discovery by the group of Keller that reduced RKIP expression may play a critical role in metastatic processes.^{57,64} Downregulation of RKIP was observed in metastatic prostate as compared to non-metastatic cell lines. Immunohistochemical staining showed moderate to high RKIP expression in normal prostate and primary prostate tumors, whereas low or undetectable levels were observed in metastatic foci. Most importantly, restoration of RKIP reduced spontaneous lung metastasis in a mouse xenograph model.⁵⁷ More recent work by the Rosner group elucidated a mechanism by which invasion and metastasis is suppressed through a signaling cascade involving MAPK, the proto-oncogene MYC, the RNA-binding protein LIN28, the let-7 micro RNA, and downstream let-7 targets.⁶⁴ RKIP has also been implicated in the metastasis of breast and ovarian cancers.^{65,66}

RKIP has also been found to be a promising prognostic marker in prostate,⁶⁷ breast,⁵⁸ and colon cancer.⁵⁹ For example, in colorectal cancer a clear prognostic relationship could be

shown between retention of RKIP expression in the primary tumor and disease free survival and mortality,⁵⁹ findings that were later confirmed in large studies by the Lugli group.^{68,69}

These studies raise the exciting possibility that drug-induced modulation of RKIP protein expression or activity may provide an avenue to control metastatic processes.^{63,70,71} Such hopes are fueled by observations that although RKIP protein expression is clearly reduced in many advanced stage tumors, it is seldom completely absent, and in some cases the reduction in expression seems to be achieved by transcriptional mechanisms.⁷² This raises the possibility that drugs could be developed to either reverse the downregulation of the RKIP promoter or to augment the remaining activity of the protein.

VI. OTHER ROLES OF PEBP PROTEINS

In addition to its role in the regulation of intracellular signaling pathways, PEBP has accumulated an unusually large number of additional putative biological functions: protease inhibitor, peptide neurotransmitter, sperm capacitance regulator, odorant receptor, and cuticle component involved in host defense (Table 1). Its signaling role has been the most thoroughly investigated, points to a function as a modulator of protein kinase activity and is consistent with wide-ranging examples from the literature, such as mammalian RKIP, plant TFL1, and yeast TFS1. PEBP proteins are typically expressed at high levels, which may lead to the possibility of artifacts due to contamination of biological preparations with PEBP. However, this is an unlikely explanation in many cases, where the same “alternative” role has been reported multiple times, and/or by different groups. It is thus likely that PEBP has indeed assumed several diverse biological roles, although unfortunately these leads have not been extensively explored.

Given its very wide phylogenetic distribution, one possible explanation is that PEBP orthologs have evolved to assume different specialized roles in different species. This may be the case with the fruit fly odorant receptor and nematode cuticle component roles, where in both cases the relevant genes encode signal sequences that would enable the transport of the proteins to their extracellular sites of action. This cannot be a universal explanation, however, because in mammals (the most thoroughly studied species), four distinct roles have been reported (Table 1, first four rows). In the case of the I_c protease inhibitor, the binding to the protease is very tight (in the nanomolar range) and the structure of the protease-PEBP complex has been solved at high resolution showing significant and specific contacts.⁷³ However, the physiological significance of this mechanism remains elusive.¹⁵

The other two mammalian examples, sperm capacitance regulator and peptide neurotransmitter (HCNP), face the additional complication that neither gene encodes a signal sequence, calling into question the extracellular localization of the protein. In the case of HCNP, a protease capable of generating mature HCNP from PEBP has been identified.⁷⁴ HCNP is subsequently presumably exported in vesicles, but the specific mechanisms have not been elucidated. There are well over 30 papers from numerous groups on HCNP, and it has been repeatedly identified in proteomic screens of brain as well as other tissues; thus, the existence of the peptide appears to be solid. In the case of the sperm capacitance regulator, PEBP has been shown to accumulate in the cytoplasmic droplet,⁷⁵ a structure of residual germ cell cytoplasm that is reabsorbed during maturation of the sperm in the epididymis. This is a known mechanism by which cytoplasmic proteins are transferred from the germ cell cytoplasm to the surface of mature sperm.

A mouse knockout of *Pebp1* has been generated but has unfortunately not been very informative.⁵⁵ The animals are viable and fertile, and display only mild reproductive and cognitive phenotypes. The reproductive deficits disappeared after backcrossing to C57Bl/6 background (J. Sedivy, unpublished observations). The cognitive defects have not been

examined in detail (it should be noted that the *Pebp1* gene was disrupted downstream of exon 1 that encodes HCNP, but whether the truncated gene can express the peptide has not been examined). The animals are not susceptible to spontaneous neoplasms, and a number of lines of investigation are currently underway to examine the effect of loss of RKIP in several cancer-prone models.

One explanation for the diversity of PEBP functions may lie in its binding pocket, which appears to be essential for biological activity in all cases where it has been examined. The shallow and extended conformation of the binding groove with a propensity to bind hydrophobic hydrocarbons⁷⁶ may enable the evolution of interactions with a number of distinct structures, with the successful cases then being subject to diversification and further evolution of function.

VII. CONCLUDING REMARKS

In the almost 30 years since its discovery, PEBP has intrigued an ever increasing number of investigators. Its highly diverse biological roles are a continuing puzzle, and its recent linkage to metastatic processes in human cancer makes further investigations of utmost interest and importance. What is critically needed to advance the field is further genetic studies in model systems. For example, we need a complete knockout of the gene to begin to address the relationship between the roles of HCNP and intracellular RKIP. Given the increasing awareness that RKIP functions as a “rheostat” rather than a “switch,” conditional knockouts will be needed to eliminate compensation of function during development and to address phenotypes in a tissue-specific manner. Finally, a regulatable transgene could be used to address the functional consequences of restoring RKIP expression in the context of a developing malignancy.

ABBREVIATIONS

AKT	v-akt murine thymoma viral oncogene homolog
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CDC25	cell division cycle 25 homolog
CEN	centroradialis
ERK	extracellular-signal-regulated kinase
GPCR	G protein–coupled receptor
GRB	growth factor receptor-bound protein
GRK2	G protein–coupled receptor kinase 2
HCNP	hippocampal cholinergic neurostimulating peptide
HSP90	heat shock protein 90
IKKα	I κ B kinase α
IKKβ	I κ B kinase β
INCENP	inner centromere protein
IκB	inhibitor of NF- κ B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KSR	kinase suppressor of RAS

LIN28	<i>C. elegans</i> lin-28 homolog
MAPK	mitogen activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MEK	MAPK/ERK kinase
NF-κB	nuclear factor of κ light polypeptide gene enhancer in B-cells
NIK	NF- κ B-inducing kinase
NMR	nuclear magnetic resonance
PEBP	phosphatidylethanolamine binding protein
PKC	protein kinase C
PP2A	protein phosphatase 2A
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RKIP	Raf kinase inhibitor protein
SAPK	stress-activated protein kinase
TAK	transforming growth factor β -activated kinase
TFL1	terminal flower 1

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FIGURE 1.

Roles of RKIP in intracellular signaling. **(A)** Downregulation of signaling mediated by inhibitory RKIP actions. **(B)** Activation of signaling elicited by removal of RKIP. Left: the ERK MAPK pathway. RKIP binds to activated RAF1 and inhibits its interaction with MEK, thus antagonizing MEK phosphorylation and activation. Phosphorylation of RKIP by protein kinase C (PKC) releases RKIP from RAF1. Center: the NF- κ B pathway. RKIP interacts with the activating kinases of the NF- κ B pathway: transforming growth factor β -activated kinase (TAK), I κ B kinase- α (IKK- α), I κ B kinase- β (IKK- β), and NF- κ B-inducing kinase (NIK). Release of RKIP from TAK, IKKs, and NIK (by an unknown mechanism) results in the phosphorylation and degradation of I κ B, which in turn releases NF- κ B. Right: G protein-coupled receptor (GPCR) pathways. GPCR kinase-2 (GRK2) antagonizes the activity of GPCRs. Phosphorylation of RKIP by PKC activates it for binding to GRK2, resulting in the inhibition of GRK2 activity. This allows GPCRs to interact with their cognate G-proteins (G), thus promoting GPCR-mediated signaling.

TABLE 1**Reported Functions of PBP**

Proposed Function	Localization	Signal Sequence	Examples
Signaling molecule	Intracellular	No	Mammalian RKIP, plant TFL1, yeast TFS1
Protease inhibitor	Intracellular	No	Mammalian, yeast
Peptide neurotransmitter	Extracellular	No	Mammalian HCNP
Sperm capacitance regulator	Extracellular	No	Mammalian PEBP
Odorant receptor	Extracellular	Yes	Fruit fly
Cuticle component	Extracellular	Yes	Nematode