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Review

The Molecular Functions of RalBP1 in Lung Cancer

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RalBP1 is an ATP-dependent non-ABC transporter, responsible for the major transport function in many cells including many cancer cell lines, causing efflux of glutathione-electrophile conjugates of both endogenous metabolites and environmental toxins. RalBP1 is expressed in most human tissues, and is over-expressed in non-small cell lung cancer cell lines and in many other tumor types. Blockade of RalBP1 by various approaches has been shown to increase sensitivity to radiation and chemotherapeutic drugs, leading to cell apoptosis. In xenograft tumor models in mice, RalBP1 blockade or depletion results in complete and sustained regression across many cancer cell types including lung cancer cells. In addition to its transport function, RalBP1 has many other cellular and physiological functions, based on its domain structure which includes a unique Ral-binding domain and a RhoGAP catalytic domain, as well as docking sites for multiple signaling proteins. Additionally, RalBP1 is also important for stromal cell function in tumors, as it was recently shown to be required for efficient endothelial cell function and angiogenesis in solid tumors. In this review, we discuss the cellular and physiological functions of RalBP1 in normal and lung cancer cells.

Key Words: RalBP1, Tumorigenesis, Lung cancer, R-Ras, RhoGAP

1. Introduction

2 Ral-binding protein 1 (RalBP1) has many cellular and physiological functions, based on its domain structure which includes a unique Ral-binding domain (RalBD) and a RhoGAP catalytic domain, as well as docking sites for multiple signaling proteins. As a Ral effector, RhoGAP, and adapter protein, RalBP1 has been shown to play important roles in endocytosis, mitochondrial fission, cell spreading and migration, actin dynamics during gastrulation, and Ras-induced tumorigenesis (Fig. 1). Also, RalBP1 is a non-ABC type transporter, which utilizes both of its two ATP binding sites (aa 69-74) and adjacent to the RalBD (aa 418-425) for ATPase and transport activity (Awasthi et al., 2001).

RalBP1 is expressed in most human tissues including liver, heart, ovary, lung, muscle, and kidney as well in most human tumor cell lines, and plays a crucial role in cancer (Awasthi et al., 1994; Awasthi et al., 1991; Awasthi et al., 2008; Sharma et al., 1990). Also, RalBP1 is over-expressed in multiple cancers, such as lung and ovarian carcinomas and melanomas (Awasthi et al., 1994; Awasthi et al., 1991; Awasthi et al., 2008; Sharma et al., 1990). As a prominent cellular function of RalBP1 is export of chemotherapy agents, it is a major factor in the mechanisms of drug resistance. Moreover, blockade of RalBP1 with targeting antibodies or antisense has been shown to greatly increase sensitivity to radiation and chemotherapy and lead to pronounced tumor regression in multiple types of solid tumors in mice, including xenografted tumors of cancer cells (Singhal et al., 2006; Singhal et al., 2007, Singhal et al., 2009).

In 2010, the most recent year for which detailed lung cancer statistics are available, patient survival was 20~30%

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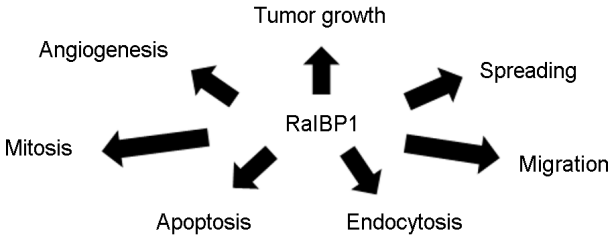

Fig. 1. RalBP1 cellular and physiological functions. RalBP1 regulates tumor growth, angiogenesis, cell spreading and migration, mitosis, apoptosis, and endocytosis in normal cells and tumor cells.

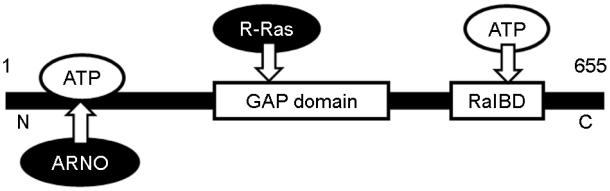

Fig. 2. The schematic diagram of RalBP1 structural domain. RalBP1 structural domains include GTPase-activating protein (GAP) domain and Ral-binding domain (RalBD), ATP binding sites, amino acid 65-80 or 415-448. RalBP1 interacts with ARNO and R-Ras through N-terminal and C-terminal regions, respectively.

(Jemal et al., 2010). Lung tumors have two major types: small cell and non-small cell lung cancer. Roughly 80~85% of lung cancers are non-small cell, and metastatic disease at presentation is common in these patients (Goldstraw et al., 2007; Riaz et al., 2012). However, response rates to chemotherapeutic regimens are low; thus, lung cancer continues to be a major cause of cancer mortality. This review will focus on the mechanisms and regulatory effects of RalBP1 in cancer and its specific roles in lung cancer.

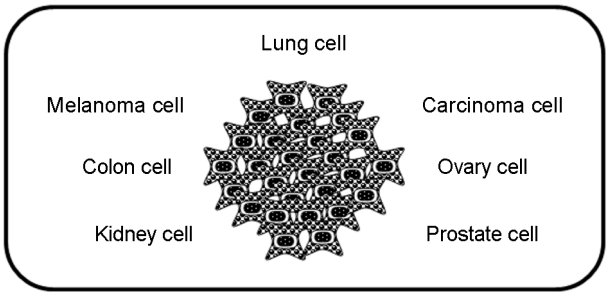

Fig. 3. RalBP1 over-expression in cancer cells. RalBP1 is over-expressed in lung, melanomas, carcinomas, colon, ovary, kidney, and prostate tumor cells.

2. RalBP1 protein

RalBP1 is a modular, 655 amino acid protein, harboring an N-terminal putative helical domain of poorly characterized function, a central RhoGAP domain on C-terminal, and a conserved RalBD near the C-terminus (Fig. 2). The RalBD, which bears no homology to classical Ras-binding domains, supports interaction with activated RalA and RalB but not with Ras small G proteins (Bauer et al., 1999; Cantor et al., 1995; Jullien-Flores et al., 1995). Like all Ras superfamily small G proteins, Ral proteins are signal transducers that become activated upon release of guanine diphosphate (GDP) and binding to guanine triphosphate (GTP), upon which Ral undergoes a conformational shift to expose high affinity binding sites for signaling effectors. Thus, RalBP1 is a unique Ral and Ras effectors, connecting upstream activation of Ral to downstream molecular and cellular events.

As shown in Fig. 3, RalBP1 is over-expressed in multiple cancers, such as lung and ovarian carcinomas and melanomas, and is expressed in most human tissues including liver, heart, ovary, lung, muscle, and kidney as well in most human tumor cell lines (Awasthi et al., 1994; Awasthi et al., 2008; Awasthi et al., 1991; Sharma et al., 1990). As a prominent cellular function of RalBP1 is export of chemotherapy agents, it is a major factor in the mechanisms of drug resistance. Also, blockade of RalBP1 with targeting antibodies or antisense has been shown to greatly increase sensitivity to radiation and chemotherapy and lead to pronounced tumor regression in multiple types of solid tumors in mice, including xenografted tumors of lung cancer cells (Singhal et al., 2006; Singhal et al., 2007; Singhal et al., 2009).

3. RalBP1 molecular function

The Ral effector, RalBP1, regulates linking Ral to Rho GTPase pathways through the RhoGAP domain (Awasthi et al., 2000; Jullien-Flores et al., 1995; Park et al., 1995). The Rho subfamily of Ras small G proteins, RhoA, Rac

and Cdc42, are regulated by guanine nucleotide exchange. Signaling by these small G proteins leads to actin remodeling and altered cell morphologies, with Rac being associated with formation of broad lamellipodia protrusions and Cdc42 with filopodia spikes (Burridege et al., 1999). Since its initial characterization as a Ral effector and RhoGAP, RalBP1 has been implicated in various other cellular functions. Also, RLIP76 supports RhoGAP activity towards Rac and Cdc42 (Jullien-Flores et al., 1995), and Ral can be activated downstream of Ras, RalBP1 was proposed to bridge Ras activation. RLIP76 interaction with RalB modulates actin remodeling during gastrulation in *Xenopus* oocytes (Lebreton et al., 2004). Thus, RalBP1 cellular and physiological functions are important with Ral effector function for Rho signaling, actin cytoskeletal remodeling, and cell morphologies.

RalBP1 has implications for the potential ATP-binding site, RalBP1 functions as an ATP-dependent glutathione-conjugate transporter for small molecules (Awasthi et al., 2000), including anticancer drugs and endogenous metabolites (Awasthi et al., 2001; Awasthi et al., 2002; Awasthi et al., 2003), and in endocytosis (Nakashima et al., 1999; Rosse et al., 2003), mitochondrial fission (Kashatus et al., 2011), cell spreading and migration (Goldfinger et al., 2006), and Ras-induced tumorigenesis (Issaq et al., 2010; Lim et al., 2005). Identified binding partners (besides RalA and RalB) include AP2, POB1, HSF-1, R-Ras, and Reps1. Through these interactions, RalBP1 participates in various cellular functions by coupling related molecules in signaling pathways. RalBP1 regulates Ral signaling in the regulation of endocytic recycling of many proteins including growth factor receptors through binding to AP2 and POB1 (Jullien-Flores et al., 2000; Nakashima et al., 1999). During mitosis, RalBP1 is phosphorylated, distributes to centrosomes and the mitotic spindle, and interacts with POB1 and mitotic kinase cdk1, leading to Ral-dependent shut off of endocytosis during mitosis (Fillatre et al., 2012; Kariya et al., 2000; Rosse et al., 2003). Additionally, during mitosis RalBP1 also localizes to mitochondria where it couples Ral phosphorylation by mitotic kinase Aurora A to signaling necessary for mitochondrial fission and proper distribution in the daughter cells (Kashatus et al., 2011). Furthermore, the role of RalBP1 in endocytosis may be coupled to its transport activity (Singhal et al., 2011). RalBP1 also appears to regulate stress-induced transcriptional activation by complexing with HSF-1 (Heat shock factor-1) (Hu et al., 2003). Moreover, RalBP1 regulates R-Ras signaling leading to cell spreading and migration (Goldfinger et al., 2006; Goldfinger et al., 2007). RalBP1 adapter function is also regulated with a small GTPase guanine exchange factor (ARNO) (Goldfinger et al., 2006), and RalBP1 potentiates Ral-mediated cell spreading, potentially through similar signaling pathways (Takaya et al., 2004). Thus, RalBP1 is regulated in many molecular, cellular and physiological processes, also its function talks to other cells as a molecular adapter.

Since its initial characterization as a Ral effector and RhoGAP, RalBP1 has been implicated in various other cellular functions, at different locations within cells, all of which are likely contributors to its efficacy as a putative cancer therapy target. RalBP1 also functions as an ATP-dependent glutathione-conjugate transporter for small molecules (Awasthi et al., 2000), including anticancer drugs and endogenous metabolites (Awasthi et al., 2001; Awasthi et al., 2002; Awasthi et al., 2003), and in endocytosis (Nakashima et al., 1999; Rosse et al., 2003), mitochondrial fission (Kashatus et al., 2011), cell spreading and migration (Goldfinger et al., 2006), and Ras-induced tumorigenesis (Issaq et al., 2010; Lim et al., 2005). RLIP76 also contains many putative sites of protein phosphorylation by several kinases such as PKCs (a family of Ser/Thr kinases), as well as Ral Interacting Kinase (RIK), and many of the utilized sites in cells are Ser and Thr residues in the N-terminal domain (Herlevsen et al., 2007; Jilkina et al., 2006). PKC-dependent RalBP1 phosphorylation appears to play an important role in its functions in lung cancer (Singhal et al., 2005; Singhal et al., 2006).

4. RalBP1 in lung cancer

RalBP1 relates to cancer progression and initiation, the most thoroughly characterized is as a molecular transporter of glutathione-electrophile conjugates (GS-E). GS-E transport is also essential for protection from xenobiotics (Cheng

et al., 2001; Srivastava et al., 1998). Multi-drug resistance (MDR), particularly for alkylating chemotherapeutic drugs, is very often the result of a failure of transport in the target cells; hence, transporters such as ATP-binding cassette (ABC) type are classified as MDR proteins, which have long been pursued as therapeutic targets to inhibit drug resistance in cancer cells (Ambudkar et al., 1999; Borst et al., 2000). Also, RalBP1 contains many putative sites of protein phosphorylation by several kinases such as Protein kinase Cs (PKCs, a family of Ser/Thr kinases), as well as Ral Interacting Kinase (RIK), and many of the utilized sites in cells are Ser and Thr residues in the N-terminal domain (Herlevsen et al., 2007; Jilkina et al., 2006). PKC-dependent RalBP1 phosphorylation appears to play an important role in its functions in cancer (Singhal et al., 2005; Singhal et al., 2006).

The various cellular functions of RalBP1, and in particularly its ATPase transport activity, have been shown to translate directly to MDR in cancer cells and in tumors of many types. Blockade of RalBP1 by anti-sense or short inhibitory RNA (siRNA) depletion, or with anti-RalBP1 antibodies (presumed to block membrane-targeted RalBP1) cause apoptosis in small cell and non-small cell lung cancer, leukemia, lymphoma, melanoma, colon cancer, and prostate cancer cell lines, and RalBP1 blockade or depletion synergizes with chemotherapeutic agents such as anthracyclines and *Vinca* alkaloids (eg, vinorelbine) to further enhance apoptosis in these cancer cell lines. These *in vitro* effects have translated in every case to pronounced *in vivo* effects in tumor xenografts: blockade of RalBP1 leads to regression of tumors formed by xenografted lung cancer, melanoma, colon cancer, prostate cancer and kidney cancer cells in mice (Sharma et al., 2004; Singhal et al., 2005; Singhal et al., 2006; Singhal et al., 2007; Singhal et al., 2009). In prostate cancer cells, anti-RalBP1 antibodies were equally as effective as siRNA, suggesting that the transport function of RalBP1 is the major driver of MDR in these cells (Singhal et al., 2009).

The role of RalBP1 as a transporter mediating MDR has been most thoroughly studied in the context of lung cancer. Early studies in this area investigated 13 native human lung cancer cell lines, and found that RalBP1 purified from non-small cell lung cancer (NSCLC) had ~ 2-fold higher ATPase activity than that from small cell lung cancer (SCLC) cell lines, perhaps due to different post-translational modifications, and RalBP1-mediated transport of doxorubicin was similarly enhanced in NSCLC compared to SCLC (Awasthi et al., 2003; Singhal et al., 2003). A recent study found RalBP1 is over-expressed in multiple NSCLC cell lines (Male et al., 2012). Another early study found that blockade of RalBP1 with specific antibodies synergized with doxorubicin to cause apoptosis in NSCLC (Awasthi et al., 2003). Subsequent studies showed that knockdown of RalBP1 by siRNA caused apoptosis in six different NSCLC cell lines, whereas augmenting RalBP1 levels led to MDR and prevention of apoptosis by both endobiotics (4-HNE) and doxorubicin (Singhal et al., 2005). Further support for RalBP1 in mediating chemotherapy resistance in lung cancer came with the finding that depletion or augmentation of RalBP1 had the same corresponding effects in both NSCLC and SCLC cells with respect to vinorelbine, a *Vinca* alkaloid with apparently less resistance in NSCLC than similar drugs (Stuckler et al., 2005). HSF-1 and POB1, binding partners of RalBP1, appear to be inhibitors of RalBP1 transport function at least in NSCLC cells, which may explain why these proteins are associated with drug sensitivity (Singhal et al., 2008; Yadav et al., 2005). However, the most direct evidence to date for a prominent role of RalBP1 in lung cancer, and its putative efficacy as a therapeutic target, was the finding that depletion of RalBP1 either with antisense or with anti- RalBP1 antibodies caused rapid and complete regression, and long-term remission of tumors in mice xenografted with two different NSCLC cell lines. Antibody and antisense treatment yielded similar results, pointing to the dominancy of the transport function in the regression, and furthermore RalBP1 blockade enhanced the regressive effects of vinorelbine in this model (Singhal et al., 2007). Thus, RalBP1 is the principal mediator of ATP-dependent transport driving efflux of chemotherapeutic agents in lung cancer cells and tumors, leading to multi-drug resistance, making RalBP1 a prominent target in developing chemotherapeutic approaches to fight lung cancer.

5. Conclusions

RalBP1 is among the most promising candidates for molecular targets in lung cancer treatment. Its multifactorial contributions to tumor growth and survival are reflected in the complete regression of lung cancer cell tumors by blocking or depleting RalBP1 in mice, and drugs targeting RalBP1 will be most useful in combinatorial therapies. Therefore, studying and understanding the molecular mechanisms of RalBP1 function could be the key to developing selective therapeutic approaches in targeting RalBP1 in lung cancer.

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