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Review

Role of RSUME in inflammation and cancer

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ABSTRACT

RSUME (for RWD-domain-containing sumoylation enhancer), *RWDD3* gene, was identified from a pituitary tumor cell with increased tumorigenic and angiogenic potential, and has higher expression in cerebellum, pituitary, heart, kidney, liver, pancreas, adrenal gland and prostate. RSUME is induced by cellular stress like hypoxia and heat shock, and is increased in pituitary tumors, in gliomas and in VHL tumors. Seven splicing forms have been described. Two of them correspond to non-coding RNAs and the other five possess an RWD domain in the N-terminus and differ in their C-terminal end. RSUME enhances SUMO conjugation by interacting with the SUMO conjugase Ubc9, increases Ubc9 thioester formation and therefore favors sumoylation of specific targets. RSUME increases IκB levels and stabilizes HIF-1α during hypoxia, leading to inhibition of NF-κB and increased HIF-1 transcriptional activity. RSUME inhibits pVHL function, thus suppressing HIF-1 and 2α ubiquitination and degradation. Disruption of the RWD domain structure of RSUME indicated that this domain is critical for RSUME action. The findings point to an important role of RSUME in the regulation and stability of specific targets, which are key regulatory mediators in cancer and inflammation.

1. Introduction

RSUME (for RWD-domain-containing sumoylation enhancer), also known as RWDD3, was cloned from the lactosomatotrophic tumor cell line GH3 overexpressing the cytokine transducer gp130 [1]. This cell line showed increased tumorigenic and angiogenic potential when injected into nude mice [2], respect to a control GH3 stable clone for the empty vector. To date, seven human RSUME mRNA splice variants have been described. Five of them code for different RSUME isoforms and two variants correspond to non-coding RNAs that suffer non-sense-mediated RNA decay, a mechanism of degradation of mRNA with premature termination codons [3,4]. The five isoforms have an RWD domain in the N-terminus and are located in the cytoplasm and nucleus, in spite of lacking a nuclear localization signal [1]. RSUME was found highly expressed in various tissues such as pituitary, cerebellum, heart, kidney, liver, pancreas, stomach, adrenal gland, prostate and spleen. It has also been associated with the development of neuropathic pain [5] and with other six genes in the molecular signature of the ganglionic eminence development [6]. Interestingly, it is upregulated by cellular stress such as hypoxia and heat shock [1,7]. Moreover, RSUME expression is increased by hypoxia in pituitary tumors [8], in the necrotic inner zone of gliomas [1] and in VHL-dependent tumors [9], making RSUME an interesting candidate for the understanding of those pathologies.

Posttranslational modification with small ubiquitin related modifier (SUMO) has been characterized as a key regulatory mechanism of protein function. SUMO belongs to the Ubiquitin family and is comprised of four distinct proteins in humans (SUMO-1, -2, -3, and -4). SUMO conjugation to the target protein regulates its function and subcellular localization, thus sumoylation governs an increasing array of cellular pathways including transport, transcription, cell cycle, DNA repair, replication and mitochondrial dynamics [10–15]. This reversible posttranslational modification is accomplished via an enzymatic cascade that consists of different steps [11,16,17]. In an ATP-dependent step, the E1-activating enzyme Aos1/Uba2 (SAE1/SAE2) forms a thioester bond between its catalytic cysteine and the C-terminal carboxy group of SUMO. In a following step, SUMO is transferred to the catalytic cysteine of the E2-conjugating enzyme Ubc9. In the last part of this cascade, an isopeptide bond is formed between SUMO and the 3-amino group of a lysine side chain, usually with the participation of E3 ligases such as Protein Inhibitor of Activated STAT (PIAS). Since it is a reversible process, specific isopeptidases, members of the SENP family, eliminate the SUMO from the target

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protein [12,17]. Conjugation/deconjugation balance seems to be a key step of regulation. Extensive progress has been accomplished in the identification of sumoylated proteins and the characterization of the consequences of the modification of specific substrates.

RSUME enhances protein sumoylation of specific targets through a direct interaction with the E2 SUMO conjugase Ubc9. A proper folding of its RWD domain is essential for RSUME activity. RSUME exerts this effect mainly in the formation of the Ubc9-SUMO-1 thioester, and also acts in the transfer of SUMO-1 from the thioester to a specific substrate [1]. Although RSUME exhibits functional similarities to SUMO ligases, it has a more general function [1].

Recently, Alontaga et al. [18] analyzed the structure of RSUME confirming, as we previously reported [1,3], that RWD is the only well-structured domain in RSUME and participates in its binding to Ubc9. Based on the crystal obtained they propose a complex with two Ubc9 and one RWD molecule, although the NMR data showed do not exclude other possible complexes. The absence of action on global sumoylation in HEK293T cells without expression of Ubc9 may be related to the critical dependence on the E2 for this RSUME action. In line with our results [1], they confirmed that RSUME stimulates Ubc9-SUMO thioester conjugate and added the stimulation of the SAE2-SUMO thioester and SAE2-SUMO isopeptide [18]. The fact that RSUME showed no effect on the sumoylation of sp100 [18], points to the notion that RSUME only acts on specific targets.

Several studies have shown that RSUME plays a key role in cancer and inflammation since it acts on specific targets related to that processes such as Hypoxia-inducible factor (HIF)-1α, von Hippel–Lindau protein (pVHL), IκB and glucocorticoid receptor (GR) [1,3,7–9] thus pointing RSUME as an interesting regulator of these pathways.

2. RSUME in hypoxia and cancer

Tumors face two major metabolic challenges: how to support enhanced cell growth and proliferation, and how to survive environmental changes in nutrient and oxygen availability when tumor growth exceeds the capacity of the existing blood vessels. Thus, reduced oxygen availability (hypoxia) stimulates metabolic adaptation in cells to promote energy production and reduce ATP consumption. This metabolic shift is synchronized by HIF-1, a transcription factor complex activated by hypoxic stress [19–21]. Under normal oxygen tension, HIF-1α accumulation is suppressed through prolyl hydroxylation (Pro402 and Pro564), leading to its ubiquitination by pVHL, which is part of the ECV complex (Elongins, Cullins and pVHL) and subsequent proteosomal degradation. During hypoxia, prolyl hydroxylation of HIF-1α is reduced, HIF-1α is not recognized by pVHL, causing HIF-1α protein stabilization, translocation to the nucleus, association with the HIF-1β subunit, and increasing HIF-1 transcriptional activity [22]. HIF-1 activity triggers the upregulation of a number of genes involved in aerobic glycolysis, including glucose transporters, glycolytic enzymes, and LDH-A, angiogenesis (such as VEGF), erythropoiesis, vascular tone and cell proliferation [22,23].

RSUME expression is induced by hypoxia and thus it might play a role in the cell's response to hypoxic stress [1]. Moreover, under hypoxic conditions, RSUME overexpression increases HIF-1α protein levels [1]. As a functional readout of HIF-1α stabilization, during hypoxia RSUME induces Vascular endothelial growth factor (VEGF) promoter activity and mRNA and protein expression, further validating the functional relevance of the interaction between RSUME and HIF [1,8]. Deletion in the RSUME promoter of the consensus sequence (RCGTG) reported to be critical for HIF-1 binding [22] abolished RSUME induction during hypoxia [1]. Furthermore, downregulation of endogenous RSUME resulted in the inhibition of endogenous HIF-1 a stabilization during hypoxia indicating an interaction between RSUME and HIF-1α [1]. Interestingly, RSUME sumoylates and physically interacts with pVHL in normoxia, and negatively regulates the assembly of the complex between pVHL, Elongins and Cullins (ECV), inhibiting HIF-1 and 2α ubiquitination and degradation [9] (Fig. 1).

As mentioned, RSUME is expressed in pituitary tumors [1,8] and gliomas [1,3], which will be further discussed below. The interplay between copy number variation (CNV) and differential gene expression may be able to shed light on molecular process underlying breast cancer and lead to the discovery of cancer-related genes. In this sense, RSUME has been associated with the gene expression signature of breast cancer patients, which display high risk of recurrence and metastases [24]. These results might have a predictive value since RSUME, as a part of this signature, could be used as biomarker with prognostic function in breast cancer. What is more, RSUME has been identified in a genome-wide association study on 2204 breast cancer patients [25]. Its relevance in VHL tumors is discussed below.

2.1. Pituitary and central nervous system tumors

Normal pituitary cells are under endocrine as well as auto-/paracrine control of numerous growth factors, so any disturbance in the expression and/or action of these components and their receptors might commit to pituitary tumor development and progression. Indeed, alterations in the expression of cytokines/growth factors and their receptors have been reported in pituitary tumors [26,27].

Pituitary tumors represent the most common intracranial neoplasms causing serious morbidity through mass effects and excessive or insufficient secretion of pituitary hormones. Radiological and autopsy studies suggest a prevalence of 22.5% [28,29].

As in any solid tumor, neovascularization is essential for pituitary adenoma progression, since the expanding tumor cell population requires nutrients and oxygen [30,31]. Furthermore, the anterior pituitary is one of the most densely vascularized mammalian organs due to the communication among hypothalamus, pituitary, and target organs, which is mediated predominantly through soluble substances transported within the bloodstream. Neovascularization is induced by angiogenesis, a process by which distinct tumor cell-derived soluble factors induce the sprouting of new vessels and ingrowth into the developing tumor [32,33]. Hypoxia triggers neovascularization through HIF, which induces the expression of different angiogenic factors such as VEGF-A, bFGF, and PDGF [27,34]. In particular, VEGF-A, which also stimulates pituitary tumor cell growth, is thought to play a key role in pituitary tumor progression, as many reports show correlations between VEGF-A and vessel density, adenoma type, size, and invasiveness [35]. In this context, RSUME may have a crucial role in this process. As mentioned, RSUME was shown to stabilize HIF-1α during hypoxia, thus inducing expression of angiogenic factors like VEGF-A [1] to trigger the formation of new vessels and, ultimately, to improve oxygen delivery under persistent hypoxic conditions. In this line, Shan et al. demonstrated that RSUME mRNA is upregulated in pituitary adenomas and significantly correlated with HIF-1α mRNA levels [8]. Hypoxia enhanced RSUME and HIF-1α expression, induced translocation of this transcription factor to the nuclei and stimulated VEGF-A production both in pituitary tumor cell lines and primary human pituitary adenoma cell cultures [8]. In addition, when RSUME expression was specifically down-regulated, VEGF-A expression was strongly reduced [8,36]. By supporting pituitary tumor neovascularization, RSUME represents a new putative therapeutic target for the development of new anti-angiogenic treatment for pituitary tumors [37].

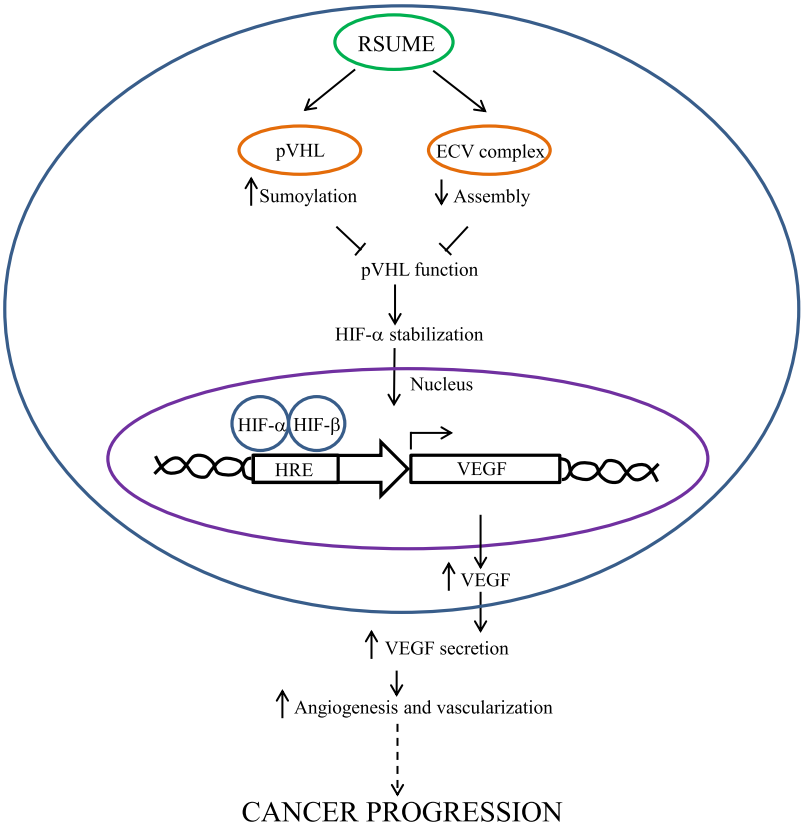

Fig. 1. RSUME stabilizes HIF-α acting mainly on pVHL and as a consequence increasing the expression of its specific targets such as VEGF that contribute to vascularization and cancer progression.

Gliomas, which represent 70% of the brain primary tumors, are characterized by extensive hypoxic areas and arise from neoplastically transformed neural stem or progenitor cells [38]. Due to the fact that they include a variety of histologic types, most glioma classifications have relied on the morphological similarities of the tumor cells with non-neoplastic glial cells and as a consequence, are classified as astrocytic, oligodendroglial, mixed oligoastrocytic, or ependymal tumors [38]. However, integrated genomic studies and exome sequencing have demonstrated the existence of multiple distinct molecular subtypes within histological classification [39] and this is fundamental for the clinical course or response to therapy. To date, only three biomarkers have been identified as potent prognostic factors in gliomas: codeletion of chromosomes 1p/19q, O6-methylguanineDNA methyltransferase (MGMT) promoter methylation, and mutations in isocitrate dehydrogenase (IDH) IDH1/2 genes [39,40].

RSUME was found to be expressed in glioma tumors. In those tumors in which two clearly different regions could be identified, Carbia-Nagashima et al. found RSUME protein expression in the necrotic inner zone but not in the peripheral zone of the tumor [1]. The mRNA and protein levels of RSUME isoforms differ in human glioma samples; while the RSUME195 isoform (195 aminoacids) is expressed in all the tumors analyzed, the longest variant (267 aminoacids) is expressed in most but not all of them [3]. These results suggest non-redundant functions of these two RSUME variants and a constitutive role of RSUME195 in the gliomas.

The fact that RSUME is induced by hypoxia in tumors and considering that it is expressed in the necrotic zone of gliomas, underscores the importance of RSUME during hypoxia in tumorigenesis. Likewise, NF-κB and HIF-1α have been proposed to interact in the regulation of angiogenesis, since cells lacking HIF-1α show an increase in NF-κB reporter activity important for the induction of IL-8 in colon cancer cells [41]. Unravelling the diverse pathways involved in angiogenesis and tumorigenesis in which sumoylation and RSUME cooperate is fundamental to develop better combined therapeutic strategies in the future.

2.2. VHL-dependent tumors

The VHL gene is involved in oxygen sensing under physiological condition and its mutations predispose carriers to devastating tumors [42]. pVHL loss-of-function mutations result in organ-specific tumors, such as hemangioblastoma of the central nervous system, renal clear-cell carcinoma (RCC) and pheochromocytoma of the adrenal gland, untreatable with conventional chemotherapies [43–45]. The pVHL protein is best known as an E3 ubiquitin ligase that targets HIF-α, but many diverse, non-canonical cellular functions have also been assigned and are still unresolved.

The presence of these diverse but specific VHL tumor types of different tissue origins is one of the intriguing facts about the VHL gene [46]. pVHL is best known as the substrate- binding subunit of a SCF (Skp1-Cdc53/Cul-1-F-box protein) type E3 ubiquitin ligase containing, besides pVHL, Cullin-2, elongin B and C and Rbx-1 [47,48]. The best-known degradation target of VHL-containing E3 ligase is HIF-α in normal physiological conditions [49,50].

By using a bioinformatic analysis in the cBioPortal for Cancer Genomics we analyzed RCC samples from the Cancer Genome Atlas Research Network and found that 4% of the tumors expressed high levels of RSUME and these patients exhibited a decrease in the survival rate [9,51–53].

Since RSUME is expressed in tumor necrotic areas and in tissues sensitive to VHL disease and given that VHL-dependent tumors

rely on very limited treatment due to the poor knowledge of the underlying mechanisms that lead to tumorigenesis, we investigated the action of RSUME on pVHL. Interestingly, RSUME stabilizes HIF-α by inhibiting pVHL. We found that RSUME increases pVHL sumoylation [9] in line with previous results showing that RSUME is a sumoylation enhancer of specific substrates, that interacts with pVHL, an interaction that is HIF-1α independent and that inhibits pVHL binding to Elongin C and Elongin B [9]. In this way, RSUME inhibits pVHL-dependent HIF-α ubiquitination (Fig. 1).

When analyzing human pVHL-deficient tumors, we obtained compelling data showing that pheochromocytoma, hemangioblastoma and RCC-786-O cell line express high levels of RSUME [9]. In this cell line, RSUME inhibits pVHL/HIF-2α interaction and acts preventing HIF-2α ubiquitination [9]. It has been shown that short hairpin RNA against RSUME (although its efficiency was not tested) did not lead to a change in the level SUMO-modified HIF-2α in hypoxic conditions [54]. In order to elucidate the contribution of the direct regulation of pVHL function by RSUME in an in vivo tumor model, we evaluated tumor formation and vascularization in nude mice injected either with RCC-786-O cells or with stable transfected clones with a short hairpin RNA for RSUME. The striking results revealed that the mice injected with clones in which RSUME was silenced, showed a significant reduction of the tumor size, lower HIF-2α and a consistent decrease in VEGF-A levels as well as in vessel density [9]. These data shed light on the critical role of RSUME on pVHL and thus, on ECV homeostasis and its impact in VHL disease tumorigenesis.

3. RSUME in inflammation

An important characteristic of the inflammatory response is the marked increase in cytokine production that activates the hypothalamic–pituitary–adrenal system, causing an increase of systemic glucocorticoid (GC) levels. GCs have a central role in the interaction between the neuroendocrine and the immune systems in order to maintain homeostasis of the whole body, avoiding excessive destruction and inflammation [55]. GCs inhibit cytokine gene expression and act as immunosuppressive and anti-inflammatory agents [55–57].

GCs are lipophilic molecules with the capability of diffusing through the cell membrane and binding the GC receptor (GR), which is a transcription factor capable of regulating either positively or negatively the expression of target genes [58–60]. Upon interaction with GCs, the GR dissociates from the complex with heat shock proteins (hsp), hsp-associated proteins, and immunophilins and translocates to the nucleus [58,59].

In the nucleus the GR binds as homodimer to specific palindromic DNA consensus sequences, the glucocorticoid response elements (GREs) by transactivation [58]. The GR can also modulate gene responses by protein–protein interaction, which is commonly responsible for repression of transcription of target genes. This mechanism is called transrepression and involves the physical association between GR and other transcription factors, such as activating protein-1 (AP-1), nuclear factor-κβ (NF-κβ), and signal transducers and activators of transcription (STAT) family members implicated in signaling of inflammatory and immunoregulatory responses [61–63]. GCs can inhibit inflammation by abrogating the activity of NF-κB, which regulates the production of proinflammatory cytokines and thus, GR activity is critical for the anti-inflammatory response [60,64].

Transcriptional activity of steroid receptors and transcription factors involved in immune and inflammatory responses may be regulated by posttranslational modifications such as phosphorylation, acetylation, prenylation, and even by covalent attachment of polypeptides, such as ubiquitin and SUMO [60]. As a result, GR regulation involves an adjustment in the inflammation process. In some cases, SUMO and ubiquitin conjugation compete for the same lysine residues on protein substrates; thus sumoylated proteins can avoid ubiquitin-mediated degradation, this being the case of the inhibitor of κβ (Iκβ) [65,66]. RSUME increases Iκβ sumoylation and stability. In addition, immunoprecipitation assays show a direct interaction between RSUME and Iκβ. Furthermore, RSUME inhibits TNF-α-induced κB-LUC (Luciferase) reporter activity, showing the functional consequence of Iκβ increased stability [1,3]. RSUME-enhanced sumoylation of Iκβ leads to the inhibition of NF-κβ activity on two well-known inflammatory genes, IL-8 and cyclooxygenase-2 (Cox-2) and therefore may also favor anti-inflammatory pathways [1] (Fig. 2).

The GR has three sumoylation sites: lysine 297 (K297) and K313 in the N-terminal domain (NTD) and K721 within the ligand-binding domain [67,68]. Interestingly, Druker et al. demonstrated that RSUME interacts with GR and increases its sumoylation, thus

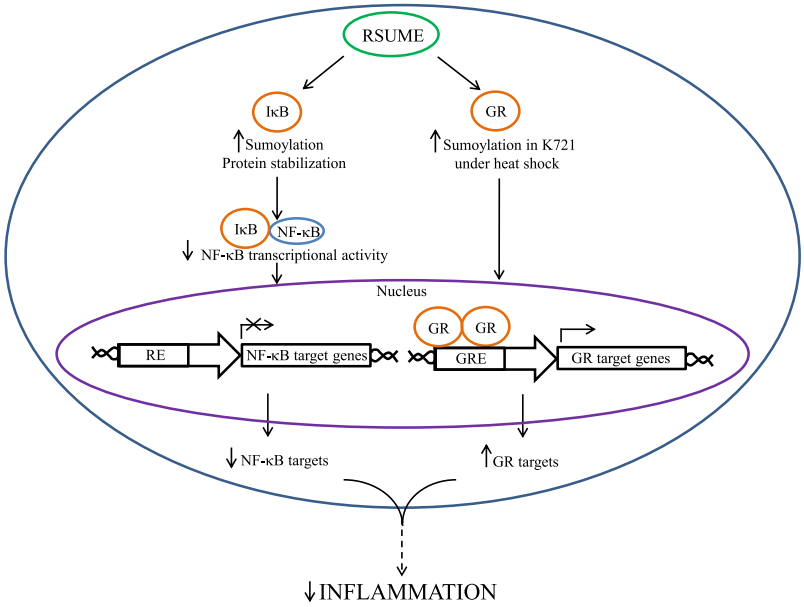

Fig. 2. RSUME stabilizes IκB and increases GR transcriptional activity thus increasing the expression of its specific targets that ultimately lead to the control of inflammation.

regulating GR transcriptional activity and the expression of its endogenous target genes, FKBP51 and S100P [7] (Fig. 2). RSUME uncovers a positive role for the third sumoylation site, K721, on GR-mediated transcription, demonstrating that GR sumoylation acts positively in the presence of this sumoylation enhancer [7]. In line with these results, mutation of K721 and small interfering RNA-mediated RSUME knockdown diminished GRIP1 coactivator activity. Given that RSUME expression is induced under stress conditions, it has a role in heat shock-induced GR sumoylation [7].

Sumoylation of the NTD sites mediates the negative effect of the synergy control motifs of GR on promoters with closely spaced GR binding sites. This third site could become relevant in a scenario of increased RSUME expression, such as under cellular stress conditions, in which RSUME can function as a molecular switch of the negative to positive action of SUMO conjugation to the GR. Understanding the actions of RSUME on specific targets involved in inflammatory responses provides further insight in the regulatory network of immune-inflammatory signals.

4. Conclusions and perspectives

Since the cloning and characterization of RSUME, several studies have pointed out its role in the regulation of specific targets. Considering that pVHL has a central role as a tumor suppressor and possesses non-canonical functions, its fine regulation through RSUME determines another important step in the regulation of HIF targets such as VEGF, with an outcome in vascularization and ultimately, to cancer progression. RSUME is also implicated in the development of pancreatic neuroendocrine tumors and acts on the tumor suppressor PTEN (U. Renner personal communication), which is frequently lost in PanNETs. This action on PTEN reinforces the role of RSUME on several specific targets related to cancer.

GRs have a pivotal action in the anti-inflammatory response and the modulation of its activity by sumoylation, particularly, an increase in its transcriptional activity by RSUME provides a novel understanding of the inflammatory pathway. In accordance, IκB stabilization by RSUME, that leads to a decrease in the expression of NF-κB targets, also contributes to the promotion of the anti-inflammatory response.

RSUME appears as an interesting player in the cross-talk of both inflammatory and hypoxic-angiogenic factors, a hypothesis and mechanism to be explored in the future.

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