Cancer Letters 339 (2013) 93–101

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

# A novel interaction between calcium-modulating cyclophilin ligand and Basigin regulates calcium signaling and matrix metalloproteinase activities in human melanoma cells

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ARTICLE INFO

*Article history:*
Received 8 May 2013
Received in revised form 11 July 2013
Accepted 15 July 2013

*Keywords:*
Basigin
CAML
Calcium
Melanoma
MMP

ABSTRACT

Intracellular free calcium is a ubiquitous second messenger regulating a multitude of normal and pathogenic cellular responses, including the development of melanoma. Upstream signaling pathways regulating the intracellular free calcium concentration ($[Ca^{2+}]i$) may therefore have a significant impact on melanoma growth and metastasis. In this study, we demonstrate that the endoplasmic reticulum (ER)-associated protein calcium-modulating cyclophilin ligand (CAML) is bound to Basigin, a widely expressed integral plasma membrane glycoprotein and extracellular matrix metalloproteinase inducer (EMMPRIN, or CD147) implicated in melanoma proliferation, invasiveness, and metastasis. This interaction between CAML and Basigin was first identified using yeast two-hybrid screening and further confirmed by co-immunoprecipitation. In human A375 melanoma cells, CAML and Basigin were co-localized to the ER. Knockdown of Basigin in melanoma cells by siRNA significantly decreased resting $[Ca^{2+}]i$ and the $[Ca^{2+}]i$ increase induced by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibitor thapsigargin (TG), indicating that the interaction between CAML and Basigin regulates ER-dependent $[Ca^{2+}]i$ signaling. Meanwhile upregulating the $[Ca^{2+}]i$ either by TG or phorbol myristate acetate (PMA) could stimulate the production of MMP-9 in A375 cells with the expression of Basigin. Our study has revealed a previously uncharacterized $[Ca^{2+}]i$ signaling pathway that may control melanoma invasion, and metastasis. Disruption of this pathway may be a novel therapeutic strategy for melanoma treatment.



## 1. Introduction

Melanoma currently accounts for only 4% of all skin cancers, but it is one of the most aggressive forms with high rates of metastasis, and the incidence is increasing worldwide [1,2]. A recent meta-analysis of 42 phase II trials for metastatic melanoma reported that the median survival time was 6.2 months and that only 25.5% of patients were still alive at one year [3].

Transient increases in the intracellular free calcium concentration ($[Ca^{2+}]i$) link extracellular signals to a myriad of cellular responses, including proliferation, accelerated metabolism, and gene transcription [4]. In cancer cells, the $Ca^{2+}$ signaling proteome might be remodeled to sustain the malignant phenotype. Carboxyamido-triazole, an inhibitor of transmembrane calcium influx and intracellular calcium-requiring signal transduction pathway, could reversibly inhibit angiogenesis, tumor cell proliferation, and metastatic potential on a broad array of human tumor cell lines [5–9]. Melanoma cells overexpressed $Ca^{2+}$-permeable chan-

*Abbreviations:* CAML, calcium modulating cyclophilin ligand; co-IP, co-immunoprecipitation; D34–89, deletion of the first IgG; D105–199, deletion of the second IgG; D207–230, deletion of the transmembrane domain; D231–269, deletion of the cytoplasmic domain; D207–269, deletion of the transmembrane and cytoplasmic domain; EMMPRIN, extracellular matrix metalloproteinase inducer; IgG, immunoglobulin; mIgG, mouse IgG; FL, full length; MMP, matrix metalloproteinase; NF-AT, the nuclear factor of activated T cells transcription factor; OPNG, ortho-nitrophenyl-β-D-galactopyranoside; PMA, phorbol myristate acetate; SERCA, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase; si-RNA, small interfering RNA; TACI, transmembrane activator and CAML-interactor; TG, thapsigargin; WCL, whole cell lysate.

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http://dx.doi.org/10.1016/j.canlet.2013.07.019

nels [10], while reducing $[Ca^{2+}]i$ or removing extracellular $Ca^{2+}$ could inhibit melanoma vasculogenic mimicry [11], or promote invasion, and proliferation [12]. Thus, signaling pathways regulating $[Ca^{2+}]i$ may trigger or suppress tumor growth and metastasis. However, these pathways and their effects on tumor aggression have not been studied in sufficient detail to facilitate the development of more effective treatments.

Calcium-modulating cyclophilin ligand (CAML) is a ubiquitous protein localized primarily to the endoplasmic reticulum (ER). It was initially described as a cyclophilin B-interacting protein that could trigger calcium influx and activate the nuclear factor of activated T cells transcription factor (NF-AT) [13]. Subsequent studies demonstrated that CAML participated in a signal transduction cascade involving a member of the tumor necrosis factor receptor superfamily, transmembrane activator and CAML-interactor (TACI) [14], to activate the transcription factors NF-AT, activator protein-1 (AP-1), and NF-κB, leading to enhanced epidermal growth factor receptor (EGFR) recycling [15]. CAML is also thought to be a signaling intermediate in numerous pathways regulating $[Ca^{2+}]i$ [13 ], protein trafficking [16] (including trafficking of postsynaptic $GABA_A$ receptors [17]), chromosome segregation [18], and lymphocyte survival [19]. Dysregulated CAML signaling has also been implicated in the pathogenesis of several diseases. CAML interacts with the renal tubulogenesis receptor fibrocystin, mutations of which lead to autosomal recessive polycystic kidney disease [20]. In addition, CAML interacts with AT1 receptor-associated protein (ATRAP), which plays an important role in cardiovascular physiology and disease [21], and with adenovirus protein E3–6.7 K, which can alter ER $Ca^{2+}$ homeostasis to protect infected cells from apoptosis. Furthermore, CAML enhances breast cancer cell proliferation by interacting with prolactin receptors [22].

Basigin (or CD147) is a widely expressed integral plasma membrane glycoprotein also known as a membrane extracellular matrix metalloproteinase inducer (termed EMMPRIN) [23,24]. It contains two Ig-like extracellular domains, a transmembrane domain, and a cytoplasmic domain. The transmembrane domain contains a highly conserved glutamic acid residue and leucine Zipper-like sequences that may allow for a variety of signaling interactions with other membrane proteins [25].

Basigin is involved in various physiological and pathophysiological processes [25–27]. The $Basigin^{-/-}$ mice are sterile, have reduced body weight, and show impaired spermatogenesis, sensory, learning, and memory functions [28]. In T cells, Basigin also plays a role during T cell development, and is a receptor for secreted cyclophilins, CypA and CypB [29]. In addition, Basigin is highly up-regulated in remodeling tissues and in many types of cancers [30,31]. Basigin was originally indentified as a factor on the surface of tumor cells that induces matrix metalloproteinase production in fibroblasts and endothelial cells, tumor cells, resulting in increased tumor invasiveness and angiogenesis [32–35]. Previous studies of our laboratory and other investigators have shown that Basigin regulates melanoma invasiveness, metastasis, cell proliferation, vascular endothelial growth factor (VEGF) production, tumor cell glycolysis, and multi-drug resistance (MDR) [36–38]. Some of these effects are thought to be mediated through activation of NO/cGMP-sensitive capacitative $Ca^{2+}$ entry [39,40]. Indeed, an anti-Basigin antibody (HAb18) strongly decreased $[Ca^{2+}]i$, while the Basigin ligand cyclophilin A had no effect on $[Ca^{2+}]i$ after HAb18 treatment [41]. However, the physiological functions of Basigin-mediated signal transduction remain largely unknown. To this end, we screened a human fetal brain cDNA library for Basigin-binding proteins using a yeast two-hybrid system. From the positive clones encoding Basigin-interacting proteins, we identified CAML. We show the Basigin interacts with CAML in A375 human melanoma cells and that this interaction may regulate ER $[Ca^{2+}]i$ signaling. In addition, increasing the $[Ca^{2+}]i$ can stimulate the production of MMP-9 in A375 cells with the expression of Basigin. These results suggest that Basigin may influence melanoma proliferation and invasive capacity by regulation of $[Ca^{2+}]i$.

## 2. Materials and methods

### 2.1. PCR and cloning

Polymerase chain reaction (PCR) was performed using the primers listed in Table 1. Diagrams of human Basigin gene (*BSG*) mutants are shown in Fig. 2A. The wild type *BSG* and deletion mutants were fused to a transcript encoding Myc, and wild type CAML was fused to a transcript encoding Flag. Deletion mutants of *BSG* and the full length (FL) CAML promoter were amplified by PCR, purified using the Cycle Pure Kit (Sangon, Shanghai, China), and digested using restriction enzymes (Takara Bio, Otsu, Japan). The pcDNA4ToA and pcDNA3ToA plasmids (Promega, WI, USA) were double digested with the same restriction enzymes. Both PCR products and plasmids were recovered from 1% agarose gels and ligated using T4 ligase (Takara Bio, Otsu, Japan) to yield pcDNA4ToA-BSG and pcDNA3ToA-CAML. After one hour incubation at room temperature, competent bacteria were transformed and plated on a selection medium containing ampicillin. Positive colonies were identified by PCR and direct sequencing.

### 2.2. Cell culture

The 293T cells were purchased from Clontech (Clontech, CA, USA), grown in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Scientific, MA, USA) supplemented with 10% FBS. The human melanoma cell line A375 cells were obtained from the American Type Culture Collection. The A375 cells expressing empty vector (A375 EV) or recombinant plasmid SUPER/Basigin short hairpin RNA (shRNA) (A375 shBasigin) had been described previously [36]. These cells were grown in RPMI 1640 medium (Thermo Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific) and 1% penicillin–streptomycin solution (Dingguo, Beijing, China). All lines were maintained in a humidified 5% $CO_2$ atmosphere at 37 °C.

**Table 1**
Primers of the PCR.

| Genes | Primers | Sequences |
|---|---|---|
| BSG | F | 5′-TAGGATCCATGGCGGCTGCGCTGTTCGTG |
| | R | 5′-CAGAATTCGGAAGAGTTCCTCTGGCGGACGTTCTTGCC |
| BSG_D34–87 | F | 5′-CACTACCGTAGAAGACCTTGTCTTCCTCCCCGAGCCC |
| | R | 5′-GGCTCGGGGAGGAAGACAAGGTCTTCTACGGTAGTG |
| BSG_D105–199 | F | 5′-TCCAGCTCCACGGGCCTCTCCGCGTGCGCAGC |
| | R | 5′-GCTGCGCACGCGGAGAGGCCCGTGGAGCTGGA |
| BSG_D207–269 | F | 5′-CGTGCGCAGCCACCTGAAGCGCCGGAAGCCCG |
| | R | 5′-CGGGCTTCCGGCGCTTCAGGTGGCTGCGCACG |
| BSG_D231–269 | R | 5′-CAGAATTCCTCGTAGATGAAGATGATGGTGACCAGC |
| BSG_D207–269 | R | 5′-CAGAATTCCAGGTGGCTGCGCACGCGGAGCG |
| CAML | F | 5′-ATGGATCCATGGAGTCGATGGCCGTCGCTAC |
| | R | 5′-GCTGACTCGAGTCATGGTACTTCAGAGCCCCA |