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Prohibitin is involved in the activated internalization and degradation of protease-activated receptor 1

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ABSTRACT

The protease-activated receptor 1 (PAR1) is a G-protein-coupled receptor that is irreversibly activated by either thrombin or metalloprotease 1. Due this irrevocable activation, activated internalization and degradation are critical for PAR1 signaling termination. Prohibitin (PHB) is an evolutionarily conserved, ubiquitously expressed, pleiotropic protein and belongs to the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain family. In a previous study, we found that PHB localized on the platelet membrane and participated in PAR1-mediated human platelet aggregation, suggesting that PHB likely regulates the signaling of PAR1. Unfortunately, PHB's exact function in PAR1 internalization and degradation is unclear. In the current study, flow cytometry revealed that PHB expressed on the surface of endothelial cells (HUVECs) but not cancer cells (MDA-MB-231). Further confocal microscopy revealed that PHB dynamically associates with PAR1 in a time-dependent manner following induction with PAR1-activated peptide (PAR1-AP), though differently between HUVECs and MDA-MB-231 cells. Depletion of PHB by RNA interference significantly inhibited PAR1 activated internalization and led to sustained Erk1/2 phosphorylation in the HUVECs; however, a similar effect was not observed in MDA-MB-231 cells. For both the endothelial and cancel cells, PHB repressed PAR1 degradation, while knockdown of PHB led to increased PAR1 degradation, and PHB overexpression inhibited PAR1 degradation. These results suggest that persistent PAR1 signaling due to the absence of membrane PHB and decreased PAR1 degradation caused by the upregulation of intracellular PHB in cancer cells (such as MDA-MB-231 cells) may render cells highly invasive. As such, PHB may be a novel target in future anti-cancer therapeutics, or in more refined cancer malignancy diagnostics.

1. Introduction

Prohibitin (PHB or PHB1), a 32 kDa protein, is ubiquitously expressed and evolutionary conserved across organisms from yeast to humans, playing roles in the regulation and maintenance of mitochondrial functions [1]. Nuclear PHB meanwhile is engaged in several important transcriptional regulations, mainly being associated with cell-cycle progression and apoptosis [2]. A number of reports over the past few years have highlighted the function of plasma membrane PHB. In human intestinal epithelial cells, for example, the complex formed by PHBs functions as a binding site for the Vi capsular polysaccharide of *Salmonella typhi*, the causative agent of typhoid fever in humans [3]. Other studies report that the membrane PHB may facilitate the entry of DENV-2 (the causative agent of the most common mosquito-borne viral disease in human) into insect cells [4]. Meanwhile, PHB engaged in cell surface Raf-MEK-ERK signaling and human platelet PAR1 signaling [5,6]. The relationship between PHB and cancer has gained increased scrutiny among researchers in recent years. Several studies on various cancer cells have found elevated protein levels of PHB [7,8]; however, the role of PHB in cancer cell remains controversial.

PAR1, the protease activated receptor 1, also known as the thrombin receptor, which belongs to the G-protein-coupled receptor family is highly expressed in a variety of cell types, including endothelial cells, platelets, monocytes, neurons and cancer cells. Consequently, PAR1 plays important roles in thrombosis, angiogenesis, inflammation and metastasis [9–11]. PAR1 is activated in a unique proteolytic manner, wherein thrombin binds to and cleaves the extracellular N-terminal

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domain of the receptor, resulting in a tethered ligand that activates PAR1 [12]. Similarly, PAR1 can be activated directly via the six-residue peptide (S/TFLLRN) that corresponds to the tethered ligand of PAR1 [13]. At this juncture, the tethered ligand cannot diffuse away, leading to activated PAR1 becoming internalized and sorted into the lysosome, resulting in its degradation. Recent investigations have demonstrated that irreversibly and proteolytically-activated PAR1 was internalized through a clathrin-and dynamin-dependent pathway and sorted to lysosome for degradation [14]. Meanwhile, experimental evidences have shown that several functional molecules participate in the regulation of PAR1 internalization and degradation, including the clathrin adaptor AP-2 [15], epsin-1 [16], ALIX [17] and sorting nexin 1 (SNX1) [18]. Additionally, constitutive internalization also plays an important role in PAR1-related functions. Unactivated PAR1 constantly cycles between the cell surface and the intracellular stores, which provides a pool that replenishes the cell surface after PAR1 activation and leads to the rapid resensitization of PAR1 signaling independent of *de novo* receptor synthesis [19].

The precise regulatory mechanisms underlying PAR1 signal termination—including internalization and degradation—are critical in the PAR1 response present in many physiological and pathological processes. Unfortunately, some aspects of the internalization and degradation of PAR1 remain unclear, especially regarding the relationship between PHB and PAR1 internalization and degradation. In a recent study, we found that PHB is localized on the platelet membrane and also involved in PAR1-mediated human platelet aggregation, indicating that PHB is a previously unknown cofactor of the PAR1-related signaling pathway [5]. For platelets which were anuclear cells, the activation of PAR1 only occurred a single time during the lifespan of platelets, suggesting that many other important events of PAR1, *e.g.*, internalization and degradation are not carried out in platelets. Previous evidence, however, found that PAR1 mainly expressed in primary cells and cancer cells [20], and likewise that PHB or PAR1 is involved the proliferation and metastasis of carcinoma cells [21]. However, there is a lack of clear evidence highlighting the different relationship between cancerous cells and either PHB or PAR1, and such information may prove useful in finding ways to overcome cancerous cell growth.

In this study, we selected two nuclear cell lines to serve as models: normal endothelial cells (HUVECs) and breast cancer cells (MDA-MB-231 cells). Our analyses of these cells showed that PHB participated in PAR1-activated internalization, Erk1/2 phosphorylation and PAR1 degradation induced by PAR1-AP in HUVECs. Meanwhile, the regulation of these processes was aberrant in MDA-MB-231 cells; showing that PHB did not regulate PAR1 activated internalization or Erk1/2 phosphorylation, but that the increased expression of PHB in cancer cells inhibited PAR1 degradation. Together, these differing properties may be responsible for the invasive capacity of different types of cancer cells, making them key targets for further research into the activities and characterization of cancerous cells.

2. Materials and methods

2.1. Materials

The PAR1-activating peptide PAR1-AP (TFLLRN) was synthesized by GL Biochem (Shanghai, China). The anti-PAR1 monoclonal antibody (ATAP2), mitochondrial marker antibody, anti-COXIV and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the anti-PHB polyclonal antibodies were purchased from R&D Systems (AF3470) (Minneapolis, MN, USA) and Santa Cruz Biotechnology (H-80) (Santa Cruz, CA, USA), and the monoclonal antibody was obtained from Neomarkers (Fremont, CA, USA). The anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody and the anti-p44/42 MAPK (Erk1/2) antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-EEA1, anti-LAMP1, anti-Histone H3 rabbit polyclonal antibodies and the mouse monoclonal M2 anti-Flag antibody were purchased from Sigma (St. Louis, MO, USA). The Alexa 488-, Alexa 594- and Alexa 647-conjugated goat anti-rabbit, goat anti-mouse and donkey anti-goat antibodies were respectively obtained from Invitrogen (Carlsbad, CA, USA). Cell mitochondria isolation and nuclear protein extraction kits were purchased from the Beyotime Institute of Biotechnology (Wuhan, Hubei, China) and the Matrigel Basement Membrane Matrix was purchased from Becton Dickinson (BD) Biosciences (San Jose, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA). Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

2.2. Calcium mobilization

To measure cytoplasmic Ca^{2+}, cells were dissociated using enzyme-free cell dissociation buffer (Invitrogen) and then incubated for 30 min with 5 μM fluo-3 AM in a buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose and 1% BSA (PH 7.4, adjust by NaOH at 37 °C). Fluo-3 fluorescence was measured at 37 °C in a Perkin-Elmer LS-5 fluorimeter at 505 nm excitation and 530 nm emission. For the cell inhibition assays, cells were pretreated with a monoclonal antibody (IV.3) to block anti-FcγRIIA (CD32) and prevent the nonspecific actions of the anti-PHB antibody.

2.3. Internalization assay

PAR1 internalization was assessed using the method previously described by Chen et al. [16]. In brief, cells were plated in 96-well dishes at a density of 5×10^3 cells per well and grown overnight. The cells were washed with PBS, and then incubated in DMEM containing 1 mg/ml BSA (pH 7.4), and subsequently either treated or not treated with 75 μM PAR1-AP for various times at 37 °C. Cells were then fixed with 4% paraformaldehyde for 5 min at 4 °C and subsequently incubated with an anti-PAR1 antibody for 1 h at 25 °C. The cells were then washed, and incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h at 25 °C. Next, the cells were washed and incubated with the horseradish peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) for 10 min, and equal aliquots were removed to new 96-well dishes. Optical density of the cells was determined at 450 nm using an Infinite M200 PRO Microplate Reader (TECAN Company, Switzerland).

2.4. Flow cytometry

The flow cytometry methods used in the present study are similar to those mentioned in our previous report [5]. In brief, to detect the surface expression of PAR1 and PHB using immunofluorescence staining, human umbilical vein epithelial cells (HUVECs) and MDA-MB-231 cells were dissociated using enzyme-free cell dissociation buffer (Invitrogen), and subsequently incubated with the appropriate primary and secondary antibodies. After washing three times, all samples were analyzed using a flow cytometer (FACSVantage SE, Becton Dickinson, NJ, USA).

2.5. Confocal microscopy

Confocal microscopy analysis was performed according to the method described by Booden et al. [22]. Both HUVECs and MDA-MB-231 cells were grown on cover slips in a 24-well tissue culture plate. The cells were then washed with PBS and incubated with an anti-PAR1 antibody for 1 h at 4 °C. Cells then were washed three times with PBS and incubated, either with or without the PAR1 agonist at 37 °C for various times. Finally, cells were fixed, permeabilized, and immunostained with the appropriate primary and secondary antibodies, and the slides were observed using a confocal microscope (Olympus FV1000, Olympus Corporation, Tokyo, Japan).