Proteasome subunit beta type 1 interacts directly with Rheb and regulates the cell cycle in Cashmere goat fetal fibroblasts

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ABSTRACT
PSMB1 (proteasome subunit beta type 1) is a core component of the 20S proteasome, and based on its structure, it might have a crucial role in the transcription of certain genes. Rheb (Ras homolog enriched in brain) is a Ras-like small GTPase that acts as an upstream positive regulator of the mechanistic target of rapamycin complex 1 (mTORC1) pathway. We cloned and characterized PSMB1 (KY310590.1) to determine its function in cell cycle progression and proliferation of goat fetal fibroblasts (GFbs). Further, by yeast two-hybrid screen and coimmunoprecipitation, we confirmed that PSMB1 interacts directly with Rheb. An siRNA was designed and expressed targeting PSMB1 mRNA in GFbs and inducing cell cycle arrest. Rheb overexpression in GFbs significantly increased the number of S phase cells and growth efficiency compared with control cells. These data indicate that PSMB1 and Rheb have important functions in the cell cycle and proliferation of GFbs, indicating that their interaction governs many processes in GFbs.

KEYWORDS
Cell cycle; goat fetal fibroblasts; PSMB1; Rheb; interaction

1. Introduction

The 20S proteasome is a core particle of the ubiquitin–26S proteasome complex and has important functions in many cellular processes, regulating the levels of key molecules in cell cycle progression, antigen presentation, secretory pathways, and signal transduction (Yuan et al. 2013). PSMB1 (proteasome subunit beta type 1) is a core component of the 20S proteasome, and its structure has been studied extensively (Wang et al. 2013). PSMB1 is believed to be crucial for the transcription of certain genes (Inoue et al. 2010; Yamauchi et al. 2013), correlate with type 1 diabetes (Bradfield et al. 2011), maintain stem cell integrity of human bone marrow stromal cells (hBMSCs) (Lu et al. 2012), and be linked to the occurrence and progression of cancer (Barton et al. 2013; Jia et al. 2015). PSMB1 is also associated with cell cycle and proliferation.

Rheb (Ras homolog enriched in brain) belongs to the Ras family and was first identified in 1994 in a screen for genes that are induced in neurons in response to synaptic activity (Kang et al. 2015). Drosophila and yeast each have a single Rheb, whereas mammals have 2: Rheb1 and Rheb2/RhebL1 (Aspuria and Tamanoi 2004). Rheb1 mRNA is nearly ubiquitous, whereas Rheb2 is expressed primarily in the brain. Rheb1 predominates over Rheb2 in the regulation of mechanistic target of rapamycin complex 1 (mTORC1) in vivo (Zou et al. 2011).

Rheb is a Ras-like small GTPase that shuttles between an active GTP-bound form and an inactive GDP-bound form, the former of which activates mTOR, acting as an upstream positive regulator in the insulin/Rheb/mTOR pathway. Rheb and mTOR colocalize on lysosomes with the lysosomal marker lysosome-associated membrane protein 2 (LAMP2) (Bonneau and Parmar 2012; Dibble et al. 2012). This relocation promotes the interaction of Rheb–GTP with mTOR in a case of nutrients (Jacobs et al. 2013; Manifava et al. 2016).

Although PSMB1 and Rheb have been studied with regard to transcription, cell differentiation, and...
tumorigenesis in mammalian cells, their physiological functions have not been examined in Cashmere goat cells. In our previous work, we identified Rheb in Inner Mongolia Cashmere goat (GenBank accession number: HM569224) (Zheng et al. 2011). In the current study, we cloned the Cashmere goat PSMB1 (GenBank accession number: KY310590.1) and analyzed the relationship and function between PSMB1 and Rheb and their coding products in Cashmere goat fetal fibroblasts (GFbs). Our results indicate that PSMB1 interacts with Rheb and regulates cell cycle progression in GFbs.

2. Materials and methods

2.1. Cell culture

Inner Mongolia Cashmere GFbs were maintained as monolayer cultures in DMEM/F12 (Gibco, Paisley, PA49RF, Scotland, UK) supplemented with 10% fetal bovine serum (FBS), 100 U mL−1 penicillin G and 100 mg mL−1 streptomycin (FBS: Hyclone Laboratories, Inc., Logan, UT, USA; penicillin/streptomycin: Sigma-Aldrich, Inc., St. Louis, USA). The cultures were maintained and incubated at 37°C in humidified air with 5% CO2.

2.2. Cloning and sequencing of PSMB1 cDNA

To amplify Inner Mongolia Cashmere goat PSMB1 cDNA, a pair of specific primers (forward: 5′-GAAGATCT-TATGTTGTCCCTCCGGCTC-3′, reverse: 5′-GGAATTCT-CAGTCCTTTCGCAGAGGAAC-3′) was designed, based on the sequence of sheep PSMB1 in GenBank (XM_004011458). The PCR program was as follows: 94°C for 4 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The 10 µL PCR mixture contained 5 µL Premix LA Taq, 0.7 µL 10 mM of each of the forward and reverse primers, 0.8 µL template cDNA, and 2.8 µL deionized water. The template cDNA was reverse-transcribed using total RNA from GFbs. The PCR products were electrophoresed, and photographs were taken on a UV transilluminator (UVItec, London, UK). The PCR products were then purified, cloned into pMD-19T (Takara, Dalian, China), and sequenced. The predicted length was 726 base pairs.

2.3. Bioinformatics analysis

Cashmere goat PSMB1 cDNA and amino acid sequences were deduced using the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST/). Prediction of the open reading frames (ORFs) and theoretical molecular weight of the deduced polypeptides was performed using the Protein property calculator (www.basic.northwestern.edu/protcalc/protein.html). The protein’s isoelectric point was predicted using an online calculator (http://isoelectric.ovh.org/). The subcellular localization of PSMB1 was predicted using PSORT (http://psort.ims.u- tokyo.ac.jp/form2.html). Protein domain analysis was conducted using SMART (http://smart.embl-heidelberg.de/). Protein profile patterns were analyzed using Psite (www.softberry.com). Three-dimensional models of certain regions were generated using SWISS-MODEL Workspace (http://swissmodel.expasy.org). Various phylogenetic trees were constructed from PSMB1 DNA sequences using MEGA4.1 by neighbor-joining method.

2.4. Yeast two-hybrid analysis of protein interaction

The Yeastmaker Yeast Transformation System 2 and Matchmaker Gold Yeast Two-Hybrid System were purchased from Clontech (Clontech Laboratorie, Inc., Mountain View, CA, USA) and used per the manufacturer’s instructions. Reagents for the synthetically defined plate for prototrophs and the colorimetric screen were also obtained from Clontech. A 726-bp fragment of PSMB1 cDNA (KY310590.1) was subcloned into pGADT7 (Clontech Laboratorie, Inc.) to generate pGADT7–PSMB1 (expressing the prey protein GAL4AD–PSMB1). pGBK7–Rheb (expressing the bait protein GAL4BD–Rheb) has been described (Wang et al. 2014). pGADT7–PSMB1 and pGBK7–Rheb were co-transformed into the Y2HGold yeast strain. The cultures were plated on SD/-Leu-Trp-His/Amp+Kan+ plate; the resulting blue colonies were transferred to an SD/-Leu-Trp-His/Ade/α-Gal/X-α-Gal/Amp+Kan+ plate; the resulting blue colonies were transferred to an SD/-Leu-Trp-His/Ade/X-α-Gal/Amp+Kan+ plate; the resulting blue colonies were transferred to an SD/-Leu-Trp-His/Ade/X-α-Gal/Amp+Kan+ plate for 3–6 days at 30°C. Segregation was confirmed based on the appearance of the white and blue colonies during the subculture.

2.5. Coimmunoprecipitation

GFbs were dissolved in cell lysis buffer (Thermo, USA) and centrifuged at 15,000 rpm at 4°C for 20 min to collect total protein. The concentrations of protein lysate were measured by using the Bio-Rad protein determination method (Bio-Rad, CA, USA). Equivalent amounts of protein lysate were incubated with anti-PSMB1 (1:50) and anti-Rheb (1:50) or anti-His (1:50) (negative control antibody) and with active resin or inactive resin in Millipore catch and release spin columns. The column was incubated on a shaker at room temperature for 45 min. The eluates (including interacting proteins) were collected by centrifugation and examined by
western blot to detect PSMB1 and Rheb. Equivalent amounts of protein lysate were also analyzed by western blot as a positive control for PSMB1 and Rheb.

2.6. Construction of pRNAT–PSMB1–shRNA and pIRES2–EGFP–Rheb
A short hairpin RNA (shRNA) sequence (5′-aaCAGT-CATTGGATGTAAGTTCAAGAGACCACACTCAATGTTG3′) that harbored the siRNA that targets PSMB1 was designed based on the sequence of Cashmere goat PSMB1 (KY310590.1). A DNA fragment that encoded the PSMB1–shRNA was synthesized and then inserted into pRNAT–U6.1/Neo to generate pRNAT–PSMB1–shRNA. The Rheb overexpression vector, pIRES2–EGFP–Rheb, was generated in our previous study (Wang et al. 2014).

2.7. In vitro transfection
The plasmids pRNAT–PSMB1–shRNA and pIRES2–EGFP–Rheb were transfected into GFbs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, NM, USA) per the manufacturer’s instructions. Transfectants were selected with G418 (Hyclone Laboratories, Inc., Logan, UT, USA) for 48 h and imaged with a digital fluorescence microscope (Olympus IX71).

2.8. Quantitative real-time PCR
Quantitative real-time polymerase chain reaction (qPCR) was performed to determine the abundance of PSMB1 in GFbs of the treated groups and control. PSMB1 was amplified with the following primers: forward 5′-AGGCGCTTCTTTCCCTACT-3′ and reverse 5′-CTCCA-CATTCTGATGTTCT-3′. β-actin was amplified with the following primer pair: forward 5′-CCACTGGCATGTTG-CTGACTC-3′ and reverse 5′-TTCCTTGATGTCACGGGAC-GATT-3′. The reactions were run using the KAPA SYBR® FAST qPCR Kit Optimized for LightCycler® 480 (KAPA BIOSYSTEMS, Inc., Boston, MA, USA) according to the manufacturer’s instructions. Total RNA from two composite groups (one each from untreated and treated cells) was reverse-transcribed with oligo (dT)12-18 primer using the AMV 1st Strand cDNA Synthesis Kit (Takara Co. Ltd., China). One microliter of cDNA was amplified in 25 μL mixture that contained 10 mM forward primer (0.5 μL), 10 mM reverse primer (0.5 μL), 2 SYBR Premix Ex Taq (12.5 μL), and nuclease-free water (10.5 μL). The program comprised an initial denaturation step at 95°C for 5 min; 40 cycles at 95°C for 5 s, 54°C for 30 s, and 72°C for 20 s; and an extension at 72°C for 10 min. Three technical replicates were run. 2−ΔΔCT values were calculated to determine expression levels, and the qPCR results were analyzed by student’s t-test to compare expression between two groups.

2.9. Western blot
Control and transfected GFbs were harvested with trypsin, washed with cold phosphate-buffered saline, and lysed in cell lysis buffer. The cells were then placed on ice for 15 min and centrifuged at 13,000 rpm and 4°C for 20 min. The concentration of lysates was measured by Bradford assay (Bio-Rad Laboratories, USA). Equal amounts (40 μg) of protein were electrophoresed on 10% (w/v) sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated overnight with primary antibody overnight at 4°C. The primary antibodies were anti-PSMB1, anti-Rheb (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (Sigma-Aldrich, Inc., St. Louis, MO, USA). Peroxidase-conjugated secondary antibody was added to the membrane at room temperature for 1 h. Enhanced chemiluminescence (ECL) reagent (Amer sham) was used to detect the signals with the Western Blotting System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The bands were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA).

2.10. Cell cycle analysis
pRNAT–PSMB1–shRNA and pIRES2–EGFP–Rheb-transfected cells and the corresponding control cells were seeded into 6-well tissue culture plates at 3×105 cells per well and incubated for 48 h at 37°C. The cells were washed with cold PBS and stained with 50 mg/L propidium iodide (PI; Sigma-Aldrich, USA), and DNA content was analyzed by flow cytometry (FACS Calibur; Becton-Dickinson Co., USA).

2.11. Cell viability assay
pIRES2–EGFP–Rheb-transfected and control cells were seeded into 96-well plates at 4×103 cells per well. Then, cell proliferation was evaluated by MTT assay once per day with 3 replicates and continued for 8 days. For the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 g/L; Sigma-Aldrich, USA) was added to each well and incubated for 4 h at 37°C. Then the formazan product was dissolved with 100 μL DMSO to each well and incubated for 10 min at 37°C. MTT absorbance was measured at 490/630 nm on spectrophotometer (Thermo, Multiskan SX 353, USA).
2.12. Statistical analysis

Descriptive statistics were generated for all quantitative data and were presented as mean ± SD. Each assay was performed in triplicate. Statistical significance was defined as \( p < .05 \).

3. Results

3.1. Cloning and characterization of PSMB1 cDNA

Full length PSMB1 cDNA (KY310590.1) from Inner Mongolia Cashmere goat comprises an ORF of 726 bp, encoding 241 amino acid residues. The sequence is 99% identical to sheep PSMB1 and 95%, 89%, 89%, 87%, and 86% identical to bovine, human, chimpanzee, rat, and mouse PSMB1, respectively. To determine the phylogenetic relationships between PSMB1 in various species, the nucleotide sequences were aligned with those of other homologs, and a phylogenetic tree was constructed, based on these alignments (Figure 1). The amino acid sequence of PSMB1 was 100% identical to that of sheep PSMB1 and 94–99% identical to other homologs.

3.2. Primary and secondary structure of putative goat PSMB1 protein

We analyzed the deduced PSMB1 protein (AQMS0982.1) of Cashmere goat, which consisted of 241 amino acid residues. The sequence contains 1 putative glycosaminoglycan attachment site, 4 protein kinase C phosphorylation sites, 1 casein kinase II phosphorylation site, 5 N-myristoylation sites, 1 prenyl group binding site (CAAX box), 4 C-terminal microbody targeting signals, and 1 proteasome B-type subunit.

Goat PSMB1 was compared with homologs in other animals using Prosite (Figure 2(A)). The predicted molecular weight of unmodified Cashmere goat PSMB1 is 26.36 kDa, and the estimated isoelectric point (pI) is 7.1. The distribution of basic amino acids is as follows: 9.5% Ser, 8.7% Gly, 8.7% Leu, 6.2% Asp, 8.3% Ala, and 7.1% Val. The putative PSMB1 protein contains a proteasome domain starting at amino acid 34 and ending at position 226, that harbors a PHD domain residues 70–106, and a T-BOX domain residues 115–219 (Figure 2(B)). According to calculations in the SWISS-MODEL workspace, we obtained a three-dimensional model of goat PSMB1 (Figure 3).

3.3. PSMB1 interacts directly with Rheb

To analyze the interaction between PSMB1 and Rheb, we constructed pGADT7–PSMB1 and co-transfected it into Y2HGold yeast cells with pGBK7–Rheb. The Y2H transfectants that contained both plasmids were verified in 4 rounds of sequential screening on SD/-Leu-Trp-His/ Amp° Kan° (Figure 4(A)), SD/-Leu-Trp-His-Ade/Amp° Kan° (Figure 4(B)), SD/-Leu-Trp-His-Ade/X-α-Gal/Amp° Kan° (Figure 4(C)) and SD/-Leu-Trp-His-Ade/Aba°/X-α-Gal/ Amp° Kan° plates. A blue colony represented a true positive clone (Figure 4(D)), indicating that the interaction between PSMB1 and Rheb brought the GAL4 AD domain in close proximity to the GAL4 BD domain, forming a complete GAL4 and resulting in expression of the four reporter genes: AUR1-C, His3, ADE2, and MEL1.

To examine the interaction between PSMB1 and Rheb in GFbs, we performed co-IP with anti-PSMB1 and anti-Rheb. The positive control was total protein lysate (Figure 5(A)). The PSMB1–Rheb protein complex was detected by co-IP but not detected in the two negative control groups (Figure 5(B)), conforming that PSMB1 interacts with Rheb in GFbs.

3.4. Knockdown of PSMB1 gene induces cell cycle arrest of GFbs

To determine the function of PSMB1 in GFbs, the PSMB1–shRNA expression vector pRNAT–PSMB1–shRNA was transfected into GFbs with Lipofectamine™ 2000. Green fluorescence was examined under a fluorescent microscope and images were obtained with a digital camera. Fluorescence was observed in the transfectants after transfection for 48 h (Figure 6(A)). To determine the efficacy of PSMB1 knock down in transfected GFbs that harbored pRNAT–PSMB1–shRNA, the relative abundance of PSMB1 mRNA was measured by real-time qPCR and PSMB1 was detected by western blot. Relative PSMB1 mRNA and its protein levels in transfected GFbs was lower compared with control cells (Figure 6(B,C)), suggesting that PSMB1 was significantly down regulated \( (p < .05) \). Next, cells that were
transfected with pRNAT–PSMB1–shRNA were analyzed by flow cytometry. The percentage of cells in S phase in the PSMB1 knockdown cells was 15.85%, versus 24.24% in control cells \((p < .05)\). These results demonstrate that cell cycle was arrested in PMSB1 silenced cells (Figure 6(D)), suggesting that PMSB1 is a positive regulator of cell cycle progression in GFbs.

### 3.5. Rheb overexpression accelerates cell cycle progression and proliferation in GFbs

To measure the effects of Rheb on the cell cycle, pIRES2–EGFP–Rheb was transfected into GFbs with Lipofectamine\textsuperscript{TM} 2000, and fluorescence was observed under a fluorescent microscope 48 h after transfection (Figure 7(A)). To confirm overexpression of Rheb in transfected GFbs that harbored pIRES2–EGFP–Rheb, Rheb was detected by western blot. Rheb expression in GFb transfectants was higher compared with control cells (Figure 7(B)), suggesting that Rheb was overexpressed. Next, the transfectants were analyzed by flow cytometry. Cell cycle progression was accelerated in Rheb-overexpressing transfectants, as evidenced by an increase in the proportion of cells in S phase (Figure 7(C)). S phase cells accounted for 64.61% of transfectants versus 19.29% in control cells \((p < .05)\). This result indicated that Rheb is a positive regulator of the cell cycle.

By MTT assay, the growth rate of transfectants was higher than in control cells from days 4 to 8 (Figure 7(D)). The growth rate differed significantly between transfected and control cells on days 4, 5, 6, and 8 \((p < .05)\). These results suggest that Rheb overexpression promotes cell proliferation by accelerating cell cycle progression in GFbs.

### 4. Discussion

Many transient protein–protein interactions regulate cellular processes. Identifying and characterizing protein interactions are thus prerequisites for understanding these events on a molecular and biophysical level (Du and Chye 2013; Ferro and Trabalzini 2013). In a yeast
one‐hybrid screen of a G4KD-L1 (GLUT4 knockdown 3T3-L1) cell cDNA library, PSMB1 was identified as a binding partner of the G4KA (GLUT4 knockdown‐dependent transcriptional activation) element (Inoue et al. 2010). Yeast two‐hybrid analysis led to the identification of PSMB1 as an interactor of the oncogenic protein BCL‐3 and the binding of BCL‐3 to PSMB1 is required for its degradation through the proteasome (Keutgens et al. 2010). In our study, we detected a novel interaction between Rheb and PSMB1.

Rheb is critical in the regulation of cell growth and cell cycle progression in S. pombe and Drosophila (Yamagata et al. 1994; Patel et al. 2003). Conditional inhibition of Rheb effects the accumulation of cells in G0/G1 phase and slow growth in S. pombe. The accumulated cells are small and resemble those that are observed after nitrogen starvation (Yang et al. 2001; Chantranupong et al. 2015). When Rheb expression is inhibited by siRNA in the S2 Drosophila tissue culture cell line, G0/G1 phase cells accumulate significantly (Patel et al. 2003).
Conversely, overexpression of Rheb in S2 cells led to a significant rise in S phase cells and a slight increase in cell size (Patel et al. 2003). Rheb knockout embryonic fibroblasts are smaller and proliferate less (Goorden et al. 2011). In our study, Rheb overexpression in GFbs led to a similar significant increase in S phase cells. Further, by MTT analysis, Rheb overexpression underwent more proliferation than control cells. Our results confirm that Rheb is essential for the cell cycle progression and growth in GFbs.

Rheb is an upstream regulator of the mTORC1 signaling pathway and is involved in protein synthesis and cell growth. mTOR signaling and the ubiquitin-proteasome system (UPS) are the main regulatory pathways that modulate the synthesis and degradation of proteins in cells, respectively. Several studies have reported that mTORC1-mediated signaling events require the proteasome (Ghosh et al. 2008) and that inhibition of the proteasome blocks mTOR activity (Hutter et al. 2012; Klappan et al. 2012). Conversely, proteasome function

**Figure 5.** Interaction between PSMB1 and Rheb by co-IP. (A) Protein lysate of GFbs was used as a positive control. PSMB1 and Rheb were detected in the four positive groups (P1, P2, P3, and P4) with equal amounts of protein. (B) PSMB1 and Rheb were detected in eluates that were precipitated with anti-PSMB1 and anti-Rheb, but not in the two negative controls (N1 with inactive resin and N2 with anti-His).

**Figure 6.** PSMB1 knock down leads to cell cycle arrest. (A) Bright field image of control cells and green fluorescence of cells transfected for 48 h with pRNAT–Rheb–shRNA (200×). (B) The relative abundance of PSMB1 mRNA in transfected GFbs was decreased compared with control cells. Transcription of PSMB1 was significantly down regulated (p < .05). (C) The expression of PSMB1 was detected by western blot. PSMB1 levels in transfected GFbs was lower compared with control cells. (D) Cell cycle analysis by flow cytometry. The percentage of cells in S phase in PSMB1 knock down cells was 15.85% versus 24.24% in control cells (p < .05). Cell cycle was arrested in PSMB1 silenced GFbs.
was regulated by mTOR signaling (Osmulski and Gaczynska 2013; Zhang et al. 2015). Recent studies have suggested that these two systems are interconnected (Ackermann 2015; Zhang et al. 2015). mTOR signaling is an evolutionarily conserved pathway regulates proteasome homeostasis (Rousseau and Bertolotti 2016). Moreover, H$_2$O$_2$ promotes the ubiquitination and degradation of Rheb and reduces the activity of mTOR in GSH-depleted RAW 264.7 cells (Seo et al. 2011). In our study, we found that PSMB1 interacts with Rheb, but this relationship and its physiological significance have not been observed in other organisms. We plan to examine how the Rheb–PSMB1 interaction senses environmental cues to control cell proliferation and other processes in GFbs.

5. Conclusion

We cloned and characterized the Cashmere goat PSMB1 (KY310590.1) and analyzed its interaction with a novel protein, Rheb. PMSB1 and Rheb regulate cell cycle progression in GFbs as positive regulators. The interaction between Rheb and PSMB1 might have important functions in GFbs, necessitating an analysis of this association.

Disclosure statement

No potential conflict of interest was reported by the authors.

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