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Low expression level of OB-Rb results from constitutive translocational attenuation attributable to a less efficient signal sequence

Ilho Choi [a], Joong-Yeol Park [d], Youngsup Song [a,b,c], Seung-Yong Yoon [b,c], Eun-Ju Chang [a,b,c], Sang-Wook Kang [a,b,c,*]

[a] Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, Republic of Korea
[b] Department of Anatomy and Cell Biology, University of Ulsan College of Medicine, Seoul, Republic of Korea
[c] Cell Dysfunction Research Center, University of Ulsan College of Medicine, Seoul, Republic of Korea
[d] Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

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ABSTRACT

OB-Rb is a crucial factor for leptin signaling. This study was initially motivated by the observation that OB-Rb expression is constitutively inhibited in the early secretory pathway. Our analyses reveal that OB-Rb contains a less hydrophobic, but functionally active N-terminal signal sequence. Constitutive translocational attenuation attributable to a less efficient signal sequence proved to be a reason for low protein level of OB-Rb. By contrast, enhanced signal sequence efficiency rescues translocation and cell surface expression of OB-Rb, and eventually potentiates leptin signaling. These observations provide considerable insight into the therapeutic enhancement of OB-Rb translocation as a potential strategy for leptin resistance.

1. Introduction

Leptin receptor is a type I single-transmembrane receptor of cytokine receptor family that plays crucial role in transduction of leptin signaling [1]. To understand a nature of leptin resistance, most efforts have focused on signal transduction pathway mediated by OB-Rb that is an isoform with the longest cytoplasmic tail and most abundantly expressed in hypothalamus [2–4]. OB-Rb plays a key role in the brain via Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway [5–7]. When circulating leptin binds to OB-Rb, tyrosine kinase JAK2 coupled with OB-Rb phosphorylates STAT3. Dimerized phospho-STAT3 subsequently enters the nucleus and regulates transcription of target genes that control appetite, metabolism and energy expenditure [5,8].

However, OB-Rb is extremely low in cell surface expression level and predominantly localized at *trans*-Golgi network even in the cells transfected with its cDNA controlled by CMV promoter [9]. Constitutive internalization of OB-Rb proved to be a reason for this observation, suggesting that intracellular trafficking lead to an altered cell surface level of OB-Rb [10–12]. However, given that degradation of internalized OB-Rb is a relatively slow process [13,14], we cannot exclude the possibility that a novel post-transcriptional or -translational mechanism at early secretory pathway may regulate OB-Rb protein level.

In the mammalian cells, early secretory pathway of secretory and membrane proteins is initiated by protein translocation across the endoplasmic reticulum (ER) membrane. We have previously discovered that this process is a regulatory process, like other regulatory systems, that might be utilized by most proteins entering the ER [15,16]. More recently, we have suggested that signal sequence efficiency attributable to sequence diversity of signal peptides determines protein translocation into the ER and leads to an altered secretion and cell surface level of proteins [15,17,18]. Considered together, signal sequence-dependent constitutive translocational attenuation may be a plausible explanation for low protein level of OB-Rb.

Abbreviations: VCAM-1, vascular endotherial cell adhesion molecule-1; PrP, prion protein; Prl, prolactin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SRP, signal recognition particle; Dox, doxycycline; TMD, transmembrane domain; PK, proteinase K; RM, rough microsome

* Corresponding author at: Department of Biomedical Sciences, University of Ulsan College of Medicine, 88, Olympic-ro 43 gil, Songpa-gu, Seoul 138-736, Republic of Korea. Fax: +82 2 3010 5307.

E-mail address: swkang@amc.seoul.kr (S.-W. Kang).

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In the present study, we monitored signal sequence and translocation efficiency of OB-Rb, and determined whether signal sequence efficiency affects cell surface expression of OB-Rb. At last, we discussed about therapeutic potential of enhanced signal sequence efficiency of OB-Rb as a strategy for leptin resistance.

2. Materials and methods

2.1. Antibodies

The following primary antibodies were used for this study: anti-HA (Roche Diagnostics, Indianapolis, IN), anti-SRP54 (BD Biosciences, San Jose, CA), anti-STAT3 and pSTAT3 (Cell Signaling Technology, Boston, MA), anti-prion protein (PrP), Sec61β, TRAPα and GFP (kindly provided by Dr. R. Hegde, University of Cambridge, UK).

2.2. Molecular biology

All cDNAs used in this study were PCR-amplified with specific primers and subcloned into pcDNA-FRT/TO or -HA for biochemical studies according to standard methods. All constructs were verified by DNA sequencing (Cosmogenetech, Seoul, Korea).

2.3. In vitro analyses

All components including T1 (for transcription) and T2 (for translation) mix were described in previous report [19]. In vitro transcription, translation, protease protection, membrane sedimentation, ubiquitination assay, SRP-crosslinking experiment and sucrose gradient of nascent chain complex were performed using previously published methods [15,20].

2.4. Cell culture analyses

HeLa and Flp-In T-Rex 293 cells were purchased from the American Type Culture Collection (Manassas, VA) and Invitrogen, respectively. Flp-In inducible systems (Invitrogen) was established and maintained according to published procedures [17,18]. Hypothalamic N1 cells (mHypoE-N1) were kind gift from M. Kim (University of Ulsan, Korea). SDS–PAGE, Western bloting, immunoprecipitation and pulse-labeling experiment were carried out according to published procedures [17,18].

Exact times and conditions in each experiment are described in individual figure legends. Procedures of biotynylation assay and retroviral infection, and additional details of experimental methods can be found in Supplemental Methods.

3. Results and discussion

3.1. OB-Rb expression is post-transcriptionally inhibited at early secretory pathway

We first monitored the relative expression level of OB-Rb to those of vascular endotherial cell adhesion molecule-1 (VCAM-1) and prolactin (Prl). To exclude the possibility of transcriptional regulation of those proteins, we established Flp-In stable cells inducibly expressing C-terminal HA epitope tagged OB-Rb, VCAM-1 and Prl whose expressions are controlled by CMV promoter in the presence of doxycycline (Dox) (Fig. 1A).

Of note, in spite of similar levels of mRNA transcribed from the all three different constructs (Fig. 1B), protein level of OB-Rb was remarkably lower than those of VCAM-1 and Prl (Fig. 1C). Given that newly synthesized OB-Rb is constitutively and rapidly internalized from the cell surface [9], these observations were not surprising. However, an intriguing observation that newly synthesized OB-Rb is not even detectable at 30 min after pulse (Fig. 1D) raised possibility of post-transcriptional inhibition of OB-Rb expression at the early secretory pathway.

3.2. OB-Rb contains less hydrophobic, but functionally active N-terminal signal sequence

Hydrophobic amino acids stretch at either the N-terminal region or internal transmembrane domain (TMD) of proteins

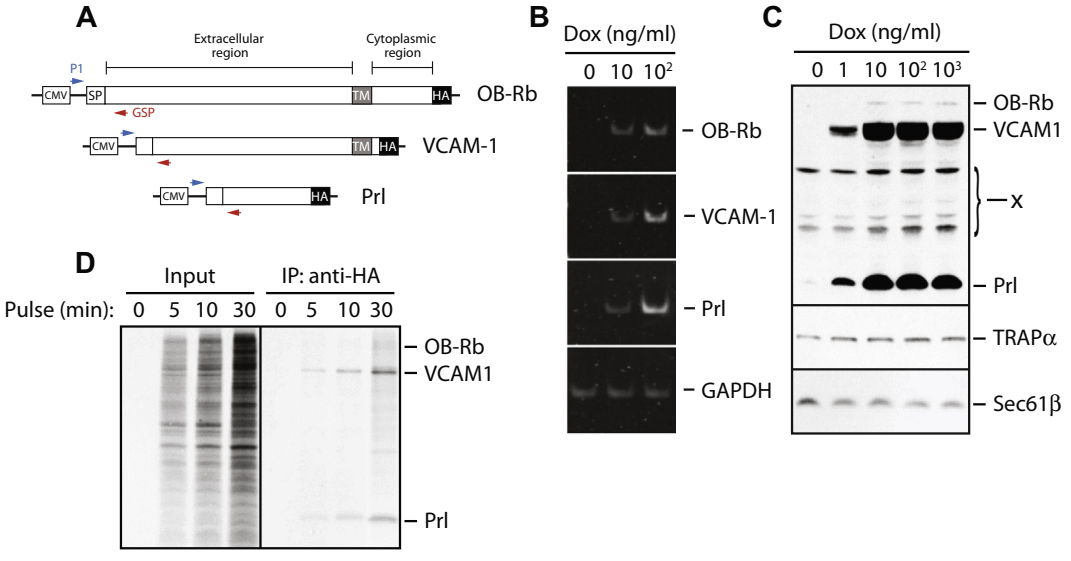

Fig. 1. Transcription-independent inhibition of OB-Rb expression. (A) C-terminal HA epitope (HA) tagged OB-Rb, VCAM-1 and Prl constructs were illustrated. Notes, "SP" = signal sequence; "CMV" = CMV promoter, "TM" = hydrophobic transmembrane domain (B) The same number of stable cells transfected with above constructs were mixed and cultured for 24 h in the presence of indicated doses of Dox. The amounts of mRNAs synthesized in the cells were measured by RT-PCR. First strand cDNAs were synthesized with oligo d(T) and subjected to PCR with a common 5′ primer annealing to 5′-UTR ("P1") and specific 3′ primers annealing to their coding regions ("GSP") as described (A). GAPDH was used as an internal control. Note, sequence information for each primer can find in Supplemental Methods. (C) The relative protein level of OB-Rb to that of VCAM-1 and Prl was monitored by Western blot analysis with anti-HA antibody. Equal protein loadings were determined with Sec61β and TRAPα levels. Note, "X" = background bands shown in the cells without induction. (D) Following incubation for 18 h in the presence of Dox (100 ng/ml), mixed stable cells were pulse-labeled with [^{35}S]-Methionine (100 μCi/ml) for the time indicated. The relative amounts of newly synthesized proteins in pulse-labeled mixed stable cells were monitored by immunoprecipitation with anti-HA antibody.

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