Cellular pathways modulating the activity of the E3 ubiquitin ligase Cbl-b in regulating T cell function

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Background

Casitas B lymphoma b (Cbl-b) is a master regulator of immune function in T cells, and represents a promising target for the immunotherapy of many diseases including cancer, type 1 diabetes, and chronic viral infection.

Cbl-b was first identified as an important regulator of T cell immunity [1, 4]. Cbl-b functions as an E3 ubiquitin ligase, and has been shown to have many target substrates including PLC-γ1, TCR-ζ[2], and Vav-1. Cbl-b is important for the maintenance of peripheral tolerance and the loss of Cbl-b results in the development of systemic autoimmunity.

Mechanistically, Cbl-b knockout (Cbl-b-/-) T cells have been shown to be refractory to TGF-β and Treg mediated suppression[3]. Cbl-b-/- T cells also bypass the requirement for CD28 co-stimulation[4]. Because of their enhanced effector activity, Cbl-b-/- CD8+ T cells were found to have increased efficacy compared to wildtype cells in mediating anti-tumor responses in mouse tumor adoptive therapy models[5]. Likewise, Cbl-b-/- mice are resistant to the development and formation of tumors compared to wildtype mice[6].

Understanding how Cbl-b is regulated could therefore provide new avenues for modulating T cell responses in vivo. Cbl-b is regulated by other E3 ligases such as Nedd4 and Itch, and also by the protein kinase PKC-θ. We aim to investigate additional signaling pathways that may regulate the activity of Cbl-b.

We have identified a putative regulatory axis for Cbl-b that involves the PI3K/Akt(PKB) pathway signaling through glycogen synthase kinase 3 (GSK-3). Using mass spectrometry and chemical inhibitors of GSK-3, we have found that the phosphorylation of Cbl-b is significantly reduced when GSK-3 activity is inhibited. Additionally, GSK-3 conditional knockout mice have reduced levels of Cbl-b in CD4+ and CD8+ T cells. We hypothesize that GSK-3 regulates Cbl-b in T cells, and that these signaling events occur downstream of the PI3K/Akt signaling pathway.

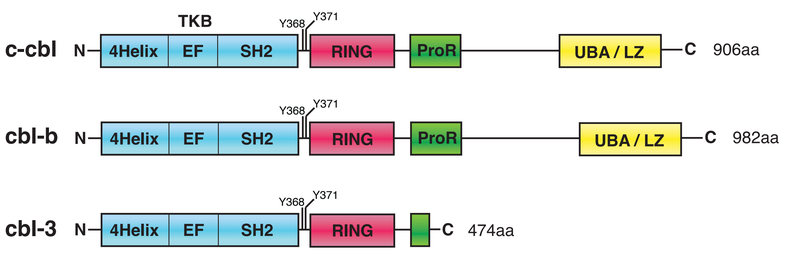

Figure 1. Domain organization of mammalian Cbl family members.

Methods

Mass spectrometry (MS)

LC-MS/MS mass spectrometry was performed using an Agilent 1100 HPLC system coupled to a hybrid LTQ-Orbitrap (ThermoQuest). MS data were submitted for MASCOT search (version 2.1; 2005) against an IPI Uni-Prot database (EBI).

In vitro T cell stimulation and Western blot

Splenocytes were isolated from wildtype, PKB transgenic, or GSK-3(αβ)fl/fl Lck Cre+ mice and purified by MACS pan T isolation (Miltenyi). Sorted T cells were stimulated in vitro with plate bound anti-CD3 and anti-CD28 antibodies for various times. Cell lysates were obtained and analyzed for Cbl-b expression by Western blot.

Results

Western blot analysis revealed reduced Cbl-b levels in both PKB transgenic T cells (Fig. 2A) and T cells treated with GSK-3 inhibitors or T cells lacking GSK-3 (Fig. 2B, 4A, 4B). Two conserved GSK-3 consensus sites were also identified in Cbl-b (Fig 3A), and only the phosphorylation of one site was altered in the presence of GSK-3 inhibitor XII (Fig. 3B) or in PKB transgenic T cells (Fig. 3C).

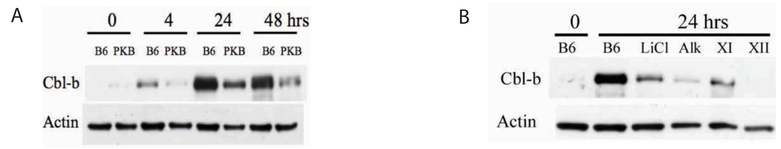

Figure 2. Cbl-b levels are reduced with increased PKB activity or GSK-3 inhibitors. (A) Reduced levels of Cbl-b in PKB transgenic T cells compared to wildtype. (B) Cbl-b levels in the presence of various GSK-3 inhibitors: lithium chloride (LiCl), 1-azakenpaullone (Alk), and compounds XI and XII.

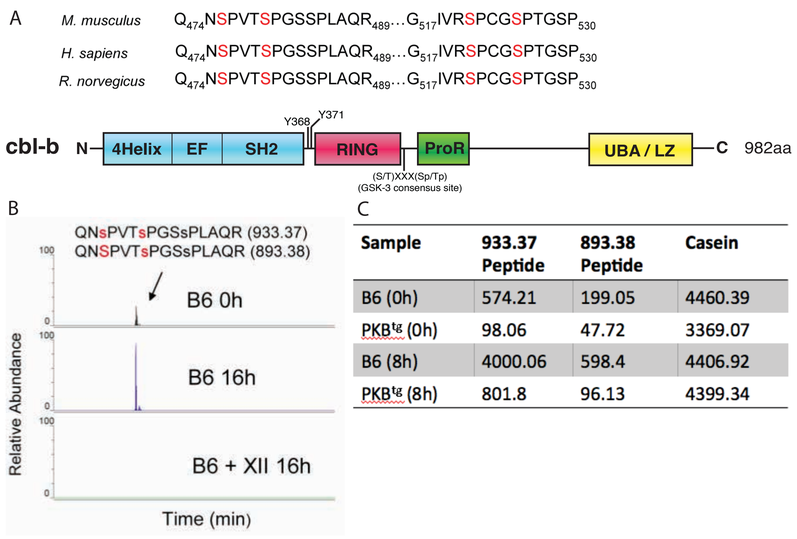

Figure 3. Protein sequence analysis and mass spectrometry of Cbl-b reveal GSK-3 to be an important regulator of Cbl-b. (A) Two conserved GSK-3 consensus sites, (S/T)XXX(S/T) were identified in Cbl-b. (B) Decreased phosphorylation of Cbl-b in stimulated T cells in the presence of a GSK-3 inhibitor, XII. (C) Reduced phosphorylation of Cbl-b in both unstimulated and stimulated PKB transgenic T cells compared to wildtype.

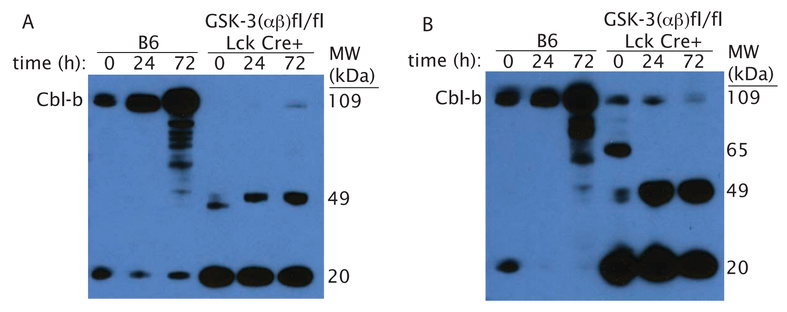

Figure 4. Cbl-b levels in B6 and GSK-3(αβ)fl/fl Lck Cre+ sorted T cells. (A) CD8+ T cells or (B) CD4+ T cells were stimulated in vitro and analyzed for Cbl-b expression at various timepoints post-stimulation.

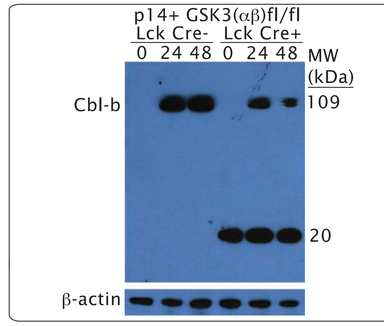

Figure 5. Reduced levels of Cbl-b in p14 TCR transgenic T cells lacking GSK-3 at various timepoints post-stimulation.

Summary

- reduced levels of Cbl-b in the presence of constitutely active PKB
- reduced levels of Cbl-b in the absence of GSK-3α and GSK-3β (knockout mice and GSK-3 inhibitors)
- in vitro data strongly suggest that GSK-3 phosphorylates Cbl-b

Discussion

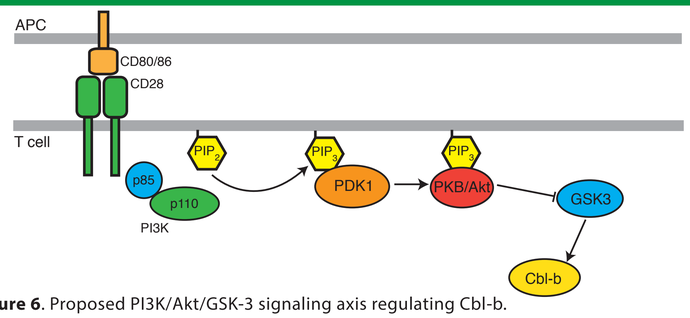

Figure 6. Proposed PI3K/Akt/GSK-3 signaling axis regulating Cbl-b.

GSK-3 is a crucial signaling molecule with key roles in metabolism and development. More recently, GSK-3 has emerged to have important functions in mediating cellular immunity[7]. Our data suggest that GSK-3 is involved in regulating the stability and turnover of Cbl-b, and this regulation appears to be downstream of the PI3K/Akt signaling pathway (Figure 6). The regulation of Cbl-b by GSK-3 represents a new potential avenue for modulating the immune response, and may find utility in cancer immunotherapy and the treatment of chronic viral infection.

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