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Research article

Working your SOCS off: The role of ASB10 and protein degradation pathways in glaucoma

Kate E. Keller*, Mary K. Wirtz

Casey Eye Institute, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

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ABSTRACT

Evidence is accumulating to suggest that mutations in the Ankyrin and SOCS Box-containing protein-10 (*ASB10*) gene are associated with glaucoma. Since its identification in a large Oregon family with primary open-angle glaucoma (POAG), ASB10 variants have been associated with disease in US, German and Pakistani cohorts. ASB10 is a member of the ASB family of proteins, which have a common structure including a unique N-terminus, a variable number of central ankyrin (ANK) repeat domains and a suppressor of cytokine signaling (SOCS) box at the C-terminus. Mutations in *ASB10* are distributed throughout the entire length of the gene including the two alternatively spliced variants of exon 1. A homozygous mutation in a Pakistani individual with POAG, which lies in the center of the SOCS box, is associated with a particularly severe form of the disease. Like other SOCS box-containing proteins, ASB10 functions in ubiquitin-mediated degradation pathways. The ANK repeats bind to proteins destined for degradation. The SOCS box recruits ubiquitin ligase proteins to form a complex to transfer ubiquitin to a substrate bound to the ANK repeats. The ubiquitin-tagged protein then enters either the proteasomal degradation pathway or the autophagic-lysosomal pathway. The choice of pathway appears to be dependent on which lysine residues are used to build polyubiquitin chains. However, these reciprocal pathways work in tandem to degrade proteins because inhibition of one pathway increases degradation via the other pathway. In this publication, we will review the literature that supports identification of *ASB10* as a glaucoma-associated gene and the current knowledge of the function of the ASB10 protein. In addition, we present new data that indicates ASB10 expression is up-regulated by the inflammatory cytokines tumor necrosis factor-α and interleukin-1α. Finally, we will describe the emerging role of other SOCS box-containing proteins in protein degradation pathways in ocular cells.

1. The *GLC1F* gene

Primary open angle glaucoma (POAG) remains a leading cause of irreversible blindness (Quigley, 2011). Identification of genes impacting glaucoma has been a long and torturous path due to the heterogeneity and complexity of the disease. Family studies utilizing pedigrees, where a large majority of relatives are afflicted, have been the standard for identifying novel genes. Using this approach, we identified a glaucoma locus, *GLC1F*, on chromosome 7q35-q36 in an Oregon family with nine individuals with glaucoma encompassing 4 generations (Wirtz et al., 1999). All affected individuals had elevated (>22 mmHg) intraocular pressure (IOP). Sequencing all of the genes in the region, a synonymous mutation in ankyrin repeat and SOCS box containing-10 (*ASB10*), T255T, was identified in all nine affected individuals (Pasutto et al., 2012). This mutation, rs104886478, has a frequency of 0.04% in the ExAc Aggregated Population. The finding that this variant is extremely rare and segregates with POAG in this family supports our hypothesis that ASB10 is a POAG susceptibility gene. Analysis of *ASB10* variants in POAG cohorts from the USA and Germany identified 26 amino acid changes in 70 patients accounting for 6% (70 of 1172) of the glaucoma population compared to 2.8% in the control group (Pasutto et al., 2012). Thus, a significant difference was found between patients and controls $P = 0.008$, two-tailed Fisher's exact test; odds ratio (OR) = 2.2, 95% confidence interval (CI) = 1.14–3.0]. The non-synonymous amino acid changes are distributed over the entire protein sequence impacting the N-terminus, the ankyrin repeats and the SOCS box.

Since our original identification, two subsequent studies have been published. In the first, eleven non-synonymous *ASB10* variants

* Corresponding author.
E-mail address: gregorka@ohsu.edu (K.E. Keller).

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were identified in a small cohort of 158 POAG patients and 82 control subjects from Iowa (Fingert et al., 2012). However, comparison of the allele frequency of each of the non-synonymous *ASB10* variants between the patients and controls showed that none was statistically more common in patients ($P > 0.05$). This paper has led to controversy over whether *ASB10* is a glaucoma-associated gene. A more recent report using a Pakistani cohort identified ten non-synonymous variants in 208 sporadic POAG patients and 151 healthy controls (Micheal et al., 2015). A burden test showed that there was a significant difference between the two groups ($p = 0.0005$). Furthermore, screening of 30 POAG families identified three rare non-synonymous variants. While these variants did not segregate with disease in the three families, one family member was homozygous for the p.Arg453Cys mutation and had a much more severe phenotype compared to his sibs. Diagnosed with POAG at the age of 15, he subsequently lost vision in both eyes at age 35 in spite of having two trabeculectomies to control the IOP of both eyes (Micheal et al., 2015). This homozygous mutation lies in the functional site of the SOCS box of ASB10 (see later).

Collectively, the three studies show that non-synonymous variants were detected in 106/1468 (7.2%) patients and in 18/694 (2.6%) controls subjects, excluding the common variants (rs62489646, rs919533 and rs201566253) (Micheal et al., 2015). Combining these studies results in a significant difference ($P = 0.000006$) in the association of rare *ASB10* variants in POAG patients compared to controls (Micheal et al., 2015). Additional population studies, as well as molecular research, will be required to determine *ASB10*'s role in glaucoma.

2. The ASB family of SOCS-box containing proteins

The family of suppressor of cytokine signaling (SOCS)-box containing proteins has over 70 members (Piessevaux et al., 2008). All SOCS-box containing proteins are involved in ubiquitination of target proteins for proteasomal-mediated protein degradation. This family is categorized into several sub-groups depending on what protein domain the SOCS box is linked to: the SOCS family, which are linked to Src homology 2 (SH2) domains; the WSB family, proteins that contain WD-40 repeats; SSB proteins, which have Spry domains; the RAR group, which have GTPase domains; and the ASB family, which contain ankyrin repeats (Hilton et al., 1998; Nicholson and Hilton, 1998). *ASB10* is one of 18 members of this sub-group (Kile et al., 2000). Members have common structural motifs including a unique N-terminal domain, a varying number of central ankyrin repeat domains and a SOCS box domain at the C-terminus. Each of these domains has distinctive functions, which we will now describe in more detail.

2.1. The N-terminus

The N-terminal region is the most divergent at the amino acid level and there is no consensus structural motif between family members. In ASB10, alternative mRNA splicing of exon 1 results in two protein isoforms called variant 1 (v1) and variant 3 (v3) (Pasutto et al., 2012). The function of the unique N-terminal regions is unknown, but POAG-associated mutations are detected in exon 1 of both of these alternatively spliced variants suggesting that they serve important biological functions (Marrs et al., 2013; Micheal et al., 2015; Pasutto et al., 2012). We performed bioinformatics analyses of the primary amino acid sequence of V1 and V3 (Fig. 1). These analyses predicted that V1 has a signal peptide and its N-terminus is likely located outside the cell. Conversely, the V3 isoform was not predicted to have a signal peptide and its N-terminus is likely located intracellularly. From these predictions, it appears that these two isoforms may perform different biological functions.

2.2. The ANK repeats

Ankyrin (ANK) repeats are one of the most common structural motifs in proteins (Mosavi et al., 2002, 2004). Each repeat, of approximately 33 residues, folds into a helix-loop-helix structure with the loop region projecting outward from the helices at a 90° angle. When multiple ANK repeats are stacked together, the loops form a concave L-shaped recognition face that binds specific proteins. The number and structure of ANK repeats contribute to the interaction with their substrates (Mosavi et al., 2002). For instance, the ANK repeats of ASB3 interact with tumor necrosis factor-alpha receptor-2 (TNFαRII), while ASB9 ANK repeats bind creatine kinase B (Chung et al., 2005; Debrincat et al., 2007). The interaction between ASB11 and ribophorin 1 appears to be dependent on the fourth of six ANK repeats (Andresen et al., 2014). Yet, for the majority of the ASB proteins including ASB10, the protein bound by the ANK repeats has not been identified.

The T255T mutation identified in the Oregon family is predicted to impact the number of ANK repeats in the resultant protein. The mutation is at the center of an exon splice enhancer site (Pasutto et al., 2012). Sequencing of *ASB10* mRNA from four of the affected family members revealed that exon 3 was skipped, which results in an altered reading frame and introduction of a stop codon in exon 4. The predicted mutant protein would have only two of the seven ankyrin repeats and the SOCS box would be completely absent. Analysis of the ASB10 protein produced from lymphoblasts from one of the affected family members showed the full-length product as well as smaller molecular weight bands (Pasutto et al., 2012). Since each of the affected individuals is heterozygous for the mutation, they have both normal and mutant ASB10 protein.

2.3. The SOCS box

The C-terminal SOCS box of approximately 40 amino acids is the most conserved motif in ASB proteins (Kile et al., 2002). The function of the SOCS box is to recruit components of the ubiquitin (Ub) ligase complex to ubiquitinate the substrate bound to the ANK repeats for degradation (Kile et al., 2002; Piessevaux et al., 2008). ASB proteins use ubiquitination to promote the turnover of their bound substrate. Thus, SOCS box-containing proteins have been implicated as negative regulators of cell signaling.

The multi-subunit Ub ligase complex include a platform protein (Cullin), a RING family protein (Rbx) and an adapter protein (elongin BC) (Kohroki et al., 2005). ASBs 1, 2, 6, 7 and 12 bind to cullin 5 (CUL5) and RING box protein-2 (RBX2) (Kohroki et al., 2005). Furthermore, a recent study used proteomics to confirm that CUL5 and RBX2 bind to ASB10 (Andresen et al., 2014). The CUL5-RBX2 ligase complex is categorized as an E3 ubiquitin ligase (Lamsoul et al., 2016). Because ubiquitination by E3 ligases control entry into the ubiquitination-mediated protein degradation pathways, these are promising targets for the development of therapeutic interventions. Several drugs targeting CUL5-RBX E3 ligases are currently in clinical trials for the treatment of some solid tumor and hematologic malignancies (Lamsoul et al., 2016; Zhao and Sun, 2013).

Primary amino acid sequence analysis of the ASB10 SOCS box shows the presence of CUL5 and Elongin BC consensus binding sequences (Fig. 2) (Kohroki et al., 2005). Interestingly, a putative POAG mutation (R438C) resides at the center of the CUL5 binding motif (Pasutto et al., 2012). Furthermore, a Pakistani individual with a particularly severe form of glaucoma was homozygous for this mutation (named R453C in their study) (Micheal et al., 2015). Because the R438C mutation sits at the center of the CUL5 binding site, it could disrupt recruitment of CUL5 to the SOCS box. It is conceivable that the substrate bound to the ANK repeats would not

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