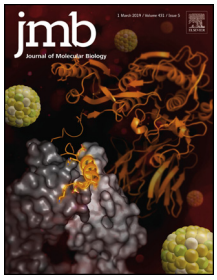

The Elusive Inhibitory Function of the Acidic N-Terminal Segment of the Prodomain of PCSK9: The Plot Thickens

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The proprotein convertase subtilisin/kexin type 9 (PCSK9) is primarily a liver-derived circulating protein representing the ninth and last member of the proprotein convertase family of mammalian secretory subtilases implicated in the generation of various bioactive proteins and peptides [1]. Like all secretory PCSK family members, in the endoplasmic reticulum (ER), proPCSK9 undergoes an autocatalytic cleavage of its N-terminal inhibitory prodomain composed of amino acids (aa) 31–152. This allows the tightly but non-covalently bound hetero-duplex of the [prodomain♦mature PCSK9] to exit the ER, traverse the Golgi apparatus and be secreted from hepatocytes into the circulation [1,2]. Thus, different from the eight other proprotein convertases that undergo separation of the active enzyme subunit from the prodomain, mature PCSK9 remains bound to its inhibitory prodomain and thus can no longer act as a protease [3].

The first functional connection of PCSK9 to LDL-cholesterol (LDLc) came from genetic analysis of autosomal dominant familial hypercholesterolemia patients who did not exhibit mutations in either the LDL-receptor (LDLR) or its ligand apolipoprotein B on LDL particles. Rather these patients exhibited gain-of-function (GOF) mutations in the prodomain (S127R) and catalytic subunit (F216L) of PCSK9 that result in higher circulating levels of LDLc [4]. Soon after, it was shown that in a Ca^{2+}-dependent manner the catalytic subunit PCSK9 binds the EGF-A domain of the cell surface LDLR on hepatocytes [5,6], and the trimeric [prodomain♦PCSK9]♦LDLR complex is then targeted to endosomes/lysosomes for degradation by an as yet unknown mechanism, which does not require the protease activity of PCSK9 [2,7–11]. It was subsequently reported that other PCSK9 missense mutations can lead to a loss-of-function (LOF) associated with lower levels of LDLc, and that the very rare individuals lacking PCSK9 (only four of them are known worldwide) exhibit very low levels of circulating LDLc (~0.4 mM) [12–14]. This led to the development and clinical use of powerful new injectable agents to reduce the levels of circulating active PCSK9 (monoclonal antibodies; mAb) or to silence the transcription of PCSK9 (siRNA) and hence drastically reduce LDLc by ~60% [2,15].

Interestingly, deletion of aa Asp_{33} to Ala_{53} of the prodomain (Δ33–53) results in a >7-fold enhanced PCSK9-binding to the EGF-A domain of the LDLR [6] and a corresponding ~3-fold enhanced degradation of cellular LDLR in hepatocytes [16]. These data suggest that the acidic N-terminal segment aa 32–54 of the prodomain (Fig. 1) inhibits the PCSK9's ability to enhance the degradation of the LDLR. Such a conclusion is further supported by some of the identified GOF mutations E32K [17], D35Y [18] and E54A that reduce the negative charges of the N-terminal prosegment, and relatively common (4.2% homozygosity) LOF variant R46L that does the inverse (Fig. 1). In addition, the chimeric replacement of the acidic stretch in PCSK9 by a similar one from GPIHBP1 resulted in a similar auto-inhibitory effect [16]. This suggested that it is the negative charge in this disordered segment that regulates such auto-inhibition and not a specific structure within. Does the acidic stretch bind the LDLR, or alternatively an inhibitory protein, or does it repel an activating one? The available 16 X-ray crystallography analyses of PCSK9 at both neutral and acidic pHs [6,19,20] revealed that the structural details of the segment comprising aa Gln_{31}–Thr_{60} (Fig. 1) are not visible in any crystal structure solved. This suggests that this highly acidic stretch rich in Glu

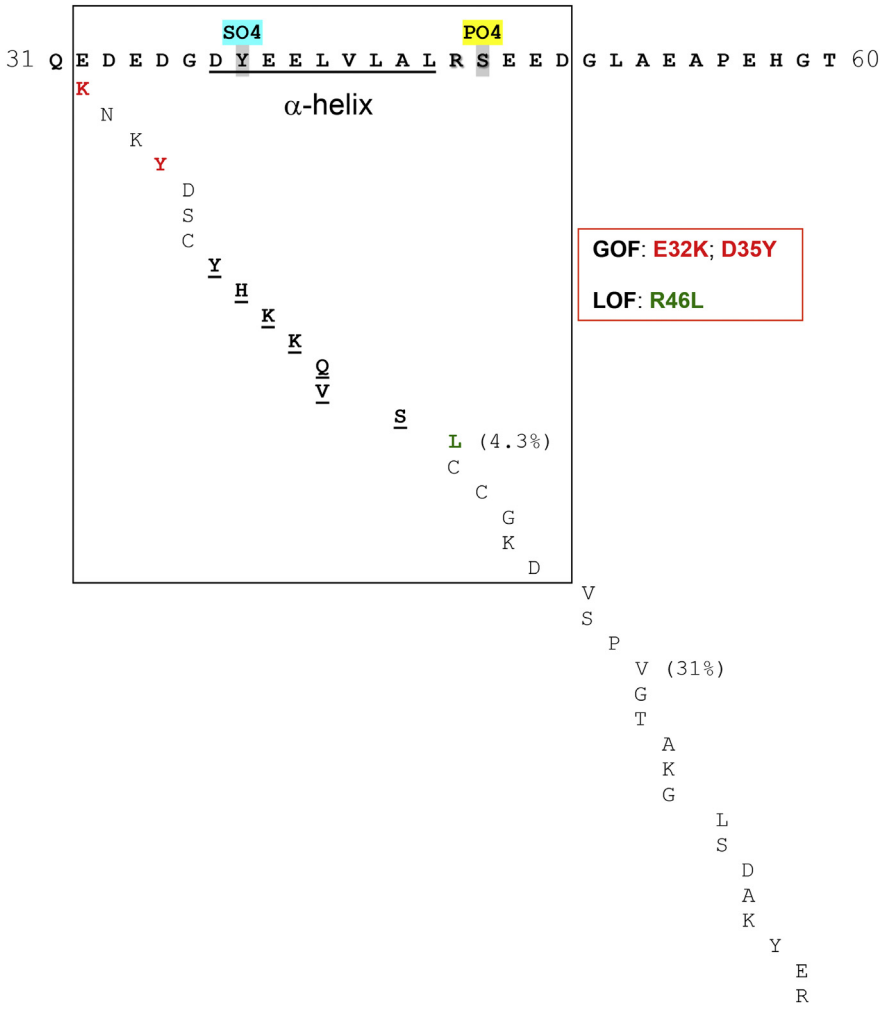

Fig. 1. Schematic representation of the N-terminal primary sequence of human PCSK9 following its signal peptide cleavage, that is from residues Gln_{31} to Thr_{60}. The reported 37 missense variants are identified under the corresponding aa. The boxed sequence covers the X-ray structurally resolved segment Glu_{32} to Asp_{50}. The underlined 9 aa Asp_{37} to Leu_{45} form the α-helix, and the missense mutations within are also underlined, including the possibly sulfated Tyr_{37} in the mutant D37Y. The % heterozygosities of the R46L (4.3%) and A53V (31%) variants are also emphasized. Finally, only the functionally validated GOF (E32K and D35Y; red residues) and LOF R46L (green residue) are emphasized and boxed.

and Asp residues (25%) represents a mobile segment, which may be stabilized *in vivo* upon binding to a putative ligand/partner. Accordingly, the available structures of PCSK9 do not allow us to pinpoint which of the above mechanisms could rationalize the negative regulation of the PCSK9 activity on the LDLR by the N-terminal acidic stretch.

A compendium of the reported 37 variants of PCSK9 in the stretch encompassing aa 31–60 is presented in Fig. 1 (modified from: (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=255738). It can be appreciated that this acidic segment of PCSK9 exhibits many single-nucleotide polymorphic variants (SNPs), which result in GOF, LOF or are neutral. Some are quite rare (e.g., the GOF E32K and D35Y), others exhibit low frequencies (e.g., the LOF R46L; 4.2% heterozygosity) or are rather common (e.g., A53V; 31% heterozygosity), and their frequency depends on ethnicity. In addition, Tyr_{38} is sulfated [10,21] and Ser_{47} is phosphorylated [22] to variable extents depending on the source of PCSK9 and likely the diet and the use of statins. For example, the mutation R46L results in a significantly lower phosphorylation of Ser_{47} [22] in the motif Ser_{47}-X-Glu_{49} recognized by the secretory kinase FAM20C [23], which maybe statin regulated [24].

In this issue, Ultsch *et al.* describe the structural characterization of peptides mimicking the N-terminal acidic stretch of PCSK9 and its mutants, a feat that has previously eluded any detailed analysis due to its intrinsically disordered state in the isolated native mature PCSK9 [19]. The authors reasoned that in

view of the mobile disordered nature of this domain, the use of a specific mAb (Ab6E2), which recognizes this sequence, may lead to a stabilized structure mimicking that in the presence of a hypothesized physiological ligand/partner *in vivo*.

Accordingly, the authors synthesized 12 peptides encompassing the segment Glu_{32} to Ala_{53} including some with the known Tyr_{38} sulfation and Ser_{47} phosphorylation. Binding, NMR and crystal structure studies revealed that the Tyr_{38} sulfation is critical for the effective Ab6E2 recognition, as the sulfate group interacts with a specific preformed pocket in the antibody paratope. Furthermore, the LOF mutation R46L completely abolished Ab6E2 binding emphasizing the importance of Arg in this interaction. Amazingly, the GOF mutation E32K resulted in a well-resolved ordered conformation of the 19 aa Glu_{32}–Asp_{50} segment when bound to Ab6E2 (Fig. 1, boxed sequence). The deduced structure revealed a central 9 aa α-helix formed by Asp_{37}-Leu_{45} that is uniquely recognized by Ab6E2 (Fig. 1, underlined sequence) surrounded by extended residues. This α-helical conformation was also critical for the binding of native mature PCSK9 to Ab6E2, suggesting that such a secondary structure in the acidic segment is a general property of PCSK9 when bound to a specific ligand/partner. However, disruption of the α-helix with specific proline substitutions therein (PCSK9–L43P:A44P) did not modify the ability of PCSK9 to enhance the degradation of cell surface LDLR, as opposed to PCSK9 lacking aa 31–53 that is ~3-fold more active than native PCSK9. Therefore, the α-helical conformation does not seem to be critical for the auto-inhibitory activity of the acidic prodomain. However, the α-helical conformation may be important to guide the post-translational sulfation at Tyr_{38} and/or phosphorylation at Ser_{47} situated within and at the C-terminus of the α-helix, respectively (Fig. 1). This suggests that the auto-inhibitory activity requires an extended, structurally disordered, negatively charged acidic stretch.

However, what is missing from this work is the analysis of whether Ab6E2 affects the activity of PCSK9 on the LDLR. Would binding of native PCSK9 to the mAb Ab6E2 result in a conformational change that leads to a more active form of extracellular PCSK9 on LDLR, as it may abrogate the auto-inhibitory activity of the acidic stretch by steric hindrance? As such, Ab6E2 may not significantly affect PCSK9 activity as it mainly contacts the hydrophobic portion of the α-helix and leaves several of the acidic residues exposed to the surface and available for interactions. In addition, the upstream and downstream acidic residues (outside the structured epitope region; Fig. 1) should remain unaltered and free to interact with basic residues of a putative PCSK9-ligand/partner. Therefore, it is plausible that PCSK9-bound Ab6E2 leaves a sufficient number of available acidic residues to promote auto-inhibition. Alternatively, engineering another N-terminal antibody or basic structure, for example, an adnectin [25], that specifically targets the N-terminal acidic residues may result in a more effective PCSK9 activator.

In conclusion, the present work provides a novel concept and an intriguing framework for future studies aimed to uncover the functional and mechanistic importance of the reported 37 missense mutations in the N-terminal acidic prodomain identified so far (Fig. 1). These include seven SNPs within the α-helix, which may disrupt its efficient formation: D37Y, Y38H that abrogates sulfation at Tyr_{38}, E39K, E40K, L41Q, L41V and A44S. It also opens the way to the future identification of an unsuspected physiological ligand/partner that may modulate PCSK9 function.

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