Studies of the autoinhibitory segment comprising residues 31–60 of the prodomain of PCSK9: Possible implications for the mechanism underlying gain-of-function mutations

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**ABSTRACT**

Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the low density lipoprotein receptor (LDLR) at the cell surface and is internalized as a complex with the LDLR. In the acidic milieu of the sorting endosome, PCSK9 remains bound to the LDLR and prevents the LDLR from folding over itself to adopt a closed conformation. As a consequence, the LDLR fails to recycle back to the cell membrane. Even though it is the catalytic domain of PCSK9 that interacts with the LDLR at the cell surface, the structurally disordered segment consisting of residues 31–60 and which is rich in acidic residues, has a negative effect both on autocatalytic cleavage and on the activity of PCSK9 towards the LDLR. Thus, this unstructured segment represents an autoinhibitory domain of PCSK9. One may speculate that post-translational modifications within residues 31–60 may affect the inhibitory activity of this segment, and represent a mechanism for fine-tuning the activity of PCSK9 towards the LDLR. Our data indicate that the inhibitory effect of this unstructured segment results from an interaction with basic residues of the catalytic domain of PCSK9. Mutations in the catalytic domain which involve charged residues, could therefore be gain-of-function mutations by affecting the positioning of this segment.

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1. Introduction

The low density lipoprotein receptor (LDLR) plays a key role in lipid metabolism by clearing cholesterol-rich low density lipoprotein (LDL) from plasma by receptor-mediated endocytosis [1]. In the acidic pH of the sorting endosome, LDL is released from the LDLR and the LDLR folds back on itself to adopt a closed conformation before being recycled back to the cell membrane [2,3]. The failure to adopt a closed conformation at acidic pH leads to ectodomain cleavage and degradation of the ectodomain in the endosomal/lysosomal tract [4–6]. As a consequence of disrupted recycling of the LDLR, the number of cell-surface LDLRs is reduced. One mechanism for disrupted recycling of the LDLR is binding of proprotein convertase subtilisin/kexin type 9 (PCSK9) to the LDLR at the cell surface [4,5,7].

PCSK9 is a 692 residue zymogen with a 30 residue signal peptide [8]. Residues 31–152 constitute the prodomain, residues 153–454 constitute the catalytic domain and residues 455–692 constitute the histidine- and cysteine-rich C-terminal domain [9,10]. The prodomain is autocatalytically cleaved off from the 74 kDa pro-PCSK9 in the endoplasmic reticulum to generate the 62 kDa mature PCSK9 [8]. However, after cleavage, the prodomain remains bound to the catalytic domain to act as a chaperone to assist protein folding and to block enzymatic activity [8]. Thus, PCSK9 is secreted as an enzymatic inactive protein.

At the cell surface PCSK9 binds to the LDLR and is internalized as a complex with the LDLR [11,12]. After internalization, PCSK9 remains bound to the LDLR and thereby prevents the LDLR from adopting a closed conformation in the sorting endosome [7]. As a consequence, the LDLR undergoes ectodomain cleavage [4]. Thus, PCSK9 reduces the number of LDLRs and is a key regulator of plasma LDL cholesterol levels. Subjects who lack PCSK9 have an increased number of LDLRs and have plasma LDL cholesterol levels that are only 10% of normal [13]. Targeting PCSK9 has therefore become a therapeutic strategy to lower plasma LDL cholesterol levels.

The activity of PCSK9 to disrupt the normal recycling of the LDLR can be modified by mutations in the PCSK9 gene [14–17]. Mutations which increase the activity of PCSK9 towards the LDLR are referred to as gain-of-function mutations and these mutations cause autosomal dominant hypercholesterolemia. Mutations which decrease the activity of PCSK9 are referred to as loss-of-function mutations and these mutations cause autosomal dominant hypocholesterolemia.

Even though it is the catalytic domain of PCSK9 that primarily interacts with the LDLR at the cell surface, there is a segment of the prodomain which negatively affects the ability of PCSK9 to bind to the LDLR. This segment consisting of residues 31–60, is structurally disordered and is characterized by a large number of acidic residues [9,10]. Deletion of residues 31–53 results in a >7-fold increased affinity of...
PCSK9 to bind to the LDLR [12]. Mutation analyses have shown that the negative effect of this prosegment on the activity of PCSK9 towards the LDLR depends on its length and its negative charge [18,19]. Thus, there does not appear to be any specific residues within this segment which negatively affects PCSK9’s function [19]. One may therefore speculate that this negatively charged prosegment may interact with positively charged residues within the catalytic or C-terminal domains to inhibit the binding of PCSK9 to the LDLR.

In this study we have performed experiments to identify the mechanism by which the negatively charged, unstructured segment comprising residues 31–60 of the prodomain, reduces the activity of PCSK9 towards the LDLR. The main strategy has been to transiently transfected HepG2 cells with various PCSK9 plasmids which encode wild-type (WT) or mutant PCSK9s with or without residues 31–52 deleted, and to study the effect on the amount of LDLRs. The segment comprising residues 31–52 was chosen for the deletion experiments because prior studies have shown that deletion of these 22 residues does not interfere with the normal cleavage of the signal peptide [12,19].

2. Materials and methods

2.1. Cell culture and transfections

HepG2 cells (European Collection of Cell Cultures, Wiltshire, UK), were cultured in Modified Eagle’s medium (Gibco, Carlsbad, CA), containing streptomycin (50 μg/ml), penicillin (50 U/ml), l-glutamine (2 mM) and 10% fetal calf serum (Invitrogen, Carlsbad, CA), in a humidified atmosphere (37 °C, 5% CO2). HepG2 cells were seeded out in collagen-coated plates (BD Biosciences, San Diego, CA) (6-well plates for Western blot analysis and 12-well plates for flow cytometric analysis) and transiently transfected with the different PCSK9-containing plasmids using Fugene HD (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Culturing of CHO T-REX cells stably transfected with the WT-LDLR plasmid has been previously described [20]. These cells are also stably transfected with a tetracycline repressor which enables tetracycline-induced expression of transgenes which contain the tetracycline operator 2 element.

2.2. Mutageneses of the PCSK9 gene

A pCMV-PCSK9-FLAG plasmid was used as a template to generate mutant PCSK9 plasmids for transfection experiments to study the role of residues 31–52 for the activity of PCSK9 towards the LDLR [19]. In order to purify WT-PCSK9 or furin-cleaved PCSK9, a pcDNA3.1-PCSK9-V5-his plasmid [21] was used as a template for mutagenesis. Mutageneses were performed using QuickChange XL Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The primers used to delete residues 31–52 of full-length mutant PCSK9 plasmids were: 5′-CCCCGCTGCCGCGCCGCCGCGCCGGCAGACAC-3′ and 5′-GTGGCGGTGGTGTCGGGCCGCGCCGGCGCGCCG-3′. The other primer sequences used for mutageneses are available upon request. The integrity of the plasmids was confirmed by DNA sequencing.

2.3. Construction of the plasmid pcDNA3.1-furin-V5/his

Total RNA was isolated from IHH cells using a blood RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. cDNA was synthesized from 1 μg RNA using AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, Santa Clara, CA). Furin was amplified from 1 μg cDNA using Q5® High-Fidelity DNA Polymerase Kit (New England Biolabs, Ipswich, MA) using the forward primer 5′-CCCGGCCACCATGGTGCCGAGC-3′ and reverse primer 5′-GTCGGAGTTGGCTCTCCGCCACGCGC-3′. The forward primer starts 29 bp upstream from the start codon which is shown in bold. The reverse primer starts at codon 791 and the underlined nucleotides are mutated to remove two stop codons. The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and then cloned into a pcDNA3.1/V5-His TOPO® vector (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions to generate the pcDNA3.1-furin-V5/his plasmid. The integrity of the plasmid was confirmed by DNA sequencing. HEK293 cells were stably transfected with the pcDNA3.1-furin-V5/his plasmid as previously described [22].

2.4. Purification of furin-cleaved PCSK9

WT-PCSK9 with a V5/his tag was isolated from media of stably transfected HEK293 cells and purified as previously described [22]. To purify furin-cleaved PCSK9, HEK293 cells stably transfected with the pcDNA3.1-furin-V5/his plasmid, were transiently transfected with the WT-PCSK9 plasmid using FuGENE HD (Roche Diagnostics GmbH, Mannheim, Germany). Media were collected 24 and 48 h after transfection and V5/his-tagged, furin-cleaved PCSK9 was purified using the protocol used for purifying WT-PCSK9 [22].

2.5. Western blot analyses

Western blot analyses of cell lysates or culture media of transiently transfected HepG2 cells were carried out as previously described [4]. Briefly, 24 h after transfection, cell lysates were run on 4–20% Tris-HCl Criterion Precast Gels (Bio-Rad, Hercules, CA) and blotted onto Immuno-Blot PVDF Membranes (Bio-Rad, Hercules, CA). For Western blot analyses to study the amount of PCSK9 in culture media, the HepG2 cells had been cultured in OptiMEM medium (Gibco, Carlsbad, CA). The membranes were immobilized with a mouse IgG anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) to detect PCSK9 synthesized from the transgenes. To study the amount of LDLRs in cell lysates, the membranes were immobilized with a rabbit polyclonal anti-LDLR antibody ( Fitzgerald Industries International, Acton, MA) which recognizes the ligand-binding domain of the LDLR. A rabbit polyclonal anti-ß-tubulin antibody (Nordic Biosite AB, Täby, Sweden) was used to detect ß-tubulin which was used as a loading control. For Western blot analysis to determine the amount of purified PCSK9 taken up by stably transfected CHO T-REX cells or HepG2 cells, a mouse anti-V5 antibody (Invitrogen, Carlsbad, CA) was used to immunostain the membranes.

2.6. Flow cytometry to determine the amount of LDL internalized

Flow cytometry was used to quantify the amount of LDL internalized in HepG2 cells transiently transfected with PCSK9-containing plasmids. The analysis was performed as previously described [14]. Briefly, 24 h after transfection the cells were washed with phosphate-buffered saline and the media were replaced with Modified Eagle’s medium containing 5 mg/ml lipoprotein-deficient serum for 24 h to induce expression of the LDLR gene. The cells were then incubated with 10 μg/ml of fluorescently labelled LDL for 2 h at 37 °C before the amount of LDL internalized was determined by flow cytometry using a FACs Canto Flow Cytometer (BD Biosciences, San Diego, CA).

3. Results

3.1. The inhibitory effect of Pro31–52 is due to its interaction with the catalytic domain

Even though the C-terminal domain of PCSK9 does not interact with the EGF-A repeat of the LDLR, it has been shown that the presence of the C-terminal domain increases the affinity of PCSK9 to bind to the LDLR at the cell surface [23]. The C-terminal domain is also required for PCSK9 to remain bound to the LDLR at acidic pH [23–25]. The underlying mechanism for this role of the C-terminal domain is that it interacts with the ligand-binding domain of the LDLR [23–25]. Thus, the negatively charged segment consisting of residues 31–52 of the prosegment of...
PCS9 (Pro31–52) could reduce the activity of PCS9 to bind to the LDLR by interfering with the interaction between the C-terminal domain of PCS9 and the ligand-binding domain of the LDLR. If so, one would expect Pro31–52 to have no effect the binding of L455X-PCS9 to the LDLR as this truncated form of PCS9 lacks the C-terminal domain.

To study whether deletion of Pro31–52 affected the binding and uptake of L455X-PCS9 by the LDLR, CHO T-REx cells stably transfected with a WT-LDLR plasmid were cultured in the presence of equal amounts of purified Δ31–52-L455X-PCS9 or L455X-PCS9. As is shown in Fig. 1b, the amount of Δ31–52-L455X-PCS9 in lysates was 5-fold higher than that of L455X-PCS9. For comparison, the amount of Δ31–52-WT-PCS9 in lysates was 3-fold higher than that of WT-PCS9. Thus, deleting Pro31–52 from L455X-PCS9 increased the uptake of L455X-PCS9 by the LDLR in a fashion similar to that of deleting Pro31–52 from WT-PCS9. These findings indicate that Pro31–52 does not exhibit its negative effect by interacting with the C-terminal domain of PCS9.

### 3.2. Effect of deleting residues 31–52 on the synthesis, autocatalytic cleavage and activity of PCS9

Because our data indicate that the negatively charged Pro31–52 interacts with positively charged structures within the promdomain or catalytic domain, one could speculate that mutations in the PCS9 gene which involve positively charged residues, could be gain-of-function mutations or loss-of-function mutations depending on how these mutations affect the positioning of Pro31–52 relative to the patch of residues of PCS9 that binds to the LDLR.

To study the role of Pro31–52 in gain-of-function mutants such as S127R-PCS9, R215H-PCS9, R218H-PCS9 and R218S-PCS9 which involve arginines, HepG2 cells were transiently transfected with mutant PCS9 plasmids encoding the respective PCS9s with or without Pro31–52 deleted. The control plasmids were empty plasmid and plasmids encoding the catalytically inactive mutant S386A-PCS9 or the gain-of-function mutant D374Y-PCS9 (Fig. 2).

These studies showed that the mutants lacking Pro31–52 were synthesized in a similar fashion to that of the respective full-length PCS9s (Fig. 2). However, as demonstrated by the decreased amounts of pro-PCS9 relative to that of the respective mature PCS9, the mutants lacking Pro31–52 were autacatically cleaved to a greater extent than the respective full-length PCS9s (Fig. 2). When Pro31–52 was deleted, there was roughly a two-fold higher amount of autacatically cleaved PCS9 in lystate relative to the total amount of PCS9.

For all mutants studied, removal of Pro31–52 made the mutants more potent towards the LDLR as demonstrated by the reduced amounts of LDLRs (Fig. 2). To confirm these findings, the effect of removing Pro31–52 from the respective mutant PCS9s was also studied by flow cytometry. HepG2 cells transiently transfected with the different plasmids were incubated with fluorescently labelled LDL and the amount of LDL internalized was used as a measure of the amount of cell-surface LDLRs. As can be seen from Fig. 3, the full-length gain-of-function mutants involving arginines, had activities towards the LDLR that were similar to that of WT-PCS9. In contrast, the D374Y-PCS9 gain-of-function mutant was markedly more active than WT-PCS9 leading to a 53% reduction in the amount of LDL internalized (Fig. 3). Deleting Pro31–52 from WT-PCS9 reduced the amount of LDL internalized by 31%. For the gain-of-function mutants S127R-PCS9, R215H-PCS9, R218H-PCS9 and R218S-PCS9, deleting Pro31–52 made these mutants approximately 40% more active than the respective full-length mutants (Fig. 3) and approached the activity of that of the full-length D374Y-PCS9 mutant. Thus, if these gain-of-function mutants involving arginines somehow led to deletion of residues 31–52 in vivo, they would have an activity similar to that of D374Y-PCS9. Deleting Pro31–52 from the D374Y-PCS9 mutant did not further increase the activity towards the LDLR (data not shown).

### 3.3. Effect of mutations in the conserved loop comprising residues 213–218

However, an alternative mechanism by which mutations R215H, R218S and R218H in the conserved, unstructured loop comprising residues 213–218 (GTRFHR218) are gain-of-function mutations, could be that they interfere with furin cleavage at RFHR218 [26]. When an optimal furin cleavage site RRRR218EL is introduced in this loop, furin cleavage is complete and the furin-cleaved mutant has no effect towards the LDLR [26]. Thus, mutations R215H, R218S and R218H could be gain-of-function mutations by abolishing furin cleavage.

Deletion of Pro31–52 did not affect furin cleavage of RRRR218EL-PCS9, as both the truncated and full-length RRRR218EL-PCS9 were completely cleaved by furin (Fig. 4). However, even though both Δ31–52RRRR218EL-PCS9 and the full-length RRRR218EL-PCS9 were completely cleaved by furin, Δ31–52RRRR218EL-PCS9 was more active towards the LDLR than the full-length RRRR218EL-PCS9 (Fig. 3). This finding could suggest that it is not furin cleavage per se that abolishes the activity of PCS9 towards the LDLR. Rather, the lack of activity could be due to the mutations introduced to generate the cleavage site. To study the effect of mutations in this conserved loop on the amount of LDLRs, we generated the mutant D374Y-PCS9 where the furin-cleavage site has been destroyed. The results showed that introducing the acidic residues DEDE218 in the conserved loop, also resulted in a loss-of-function phenotype which could not be due to furin cleavage (Fig. 3). Furthermore, it should be noted that deletion of Pro31–52
from RRRR-PCSK9 and DEDE-PCSK9, also promoted autocatalytic cleavage of the two mutants (Fig. 4).

3.4. Effect of furin-cleaved PCSK9 on the activity towards the LDLR

Because of the possibility that it was the introduction of mutations in the conserved loop to create a furin cleavage site that abolished the activity of PCSK9, we decided to purify furin-cleaved WT-PCSK9 without introducing a furin cleavage site. To purify furin-cleaved WT-PCSK9, HEK293 cells were stably transfected with a furin-encoding plasmid. These cells were then transiently transfected with WT-PCSK9 plasmid and media were collected for purification of furin-cleaved PCSK9. To study the binding of furin-cleaved PCSK9 to the LDLR, CHO T-REx cells stably expressing the WT-LDLR were incubated with equal amounts of furin-cleaved or non-furin-cleaved PCSK9, and the amounts of PCSK9 in cell lysates were determined by Western blot analysis. As can be seen from Fig. 5, significant amounts of non-furin-cleaved PCSK9 were found in cell lysates of the stably transfected CHO T-REx cells, whereas virtually no furin-cleaved PCSK9 was observed in cell lysates. Thus, it appears that furin-cleaved PCSK9 is not internalized by the LDLR. The explanation for the failure of WT-PCSK9 to reduce the amount of LDLR in these stably transfected CHO T-REx cells, is due to the very high expression of the LDLR in these cells [22].

To confirm that furin-cleaved PCSK9 does not disrupt the normal recycling of the LDLR, HepG2 cells were incubated with equal amounts of furin-cleaved or WT-PCSK9, and the amounts of LDLRs in cell lysates were determined by Western blot analysis. Whereas, WT-PCSK9 reduced the amount of LDLRs in the HepG2 cells by 60%, no effect of furin-cleaved PCSK9 was observed on the amount of LDLRs (Fig. 6). Moreover, no furin-cleaved PCSK9 could be detected in the lysate of these cells (Fig. 6).

4. Discussion

In this study we have provided further data on the role of the unstructured segment of PCSK9 comprising residues 31–52, on autocatalytic cleavage and on the activity towards the LDLR. For deletion experiments, residues 31–52 (Pro31–52) were the residues that were actually deleted in order to avoid interference with the normal cleavage of the signal peptide.

When Pro31–52 was deleted from WT-PCSK9 to generate ΔPro31–52-PCSK9, the amount of ΔPro31–52-PCSK9 internalized was 3-fold higher than that of the WT-PCSK9. This indicates a higher affinity of Δ

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**Fig. 2.** Western blot analyses of HepG2 cells transiently transfected with PCSK9-containing plasmids with or without residues 31–52 deleted. HepG2 cells were transiently transfected with empty plasmid, the WT-PCSK9 plasmid or various mutant PCSK9 plasmids with or without residues 31–52 deleted (Δ31–52). The amounts of PCSK9 in the media and in lysates were determined by Western blot analysis using an antibody against the C-terminal FLAG-tag. Pro-PCSK9 and mature PCSK9 (M-PCSK9) are indicated. The amounts of LDLRs in the lysates were determined by Western blot analysis using an antibody against the ligand-binding domain of the LDLR. β-Tubulin was used as a loading control.

**Fig. 3.** Amount of LDL internalized in HepG2 cells transiently transfected with PCSK9-containing plasmids with or without residues 31–52 deleted. HepG2 cells were transiently transfected with empty plasmid, the WT-PCSK9 plasmid or various mutant PCSK9 plasmids with or without residues 31–52 deleted (Δ31–52). The amounts of fluorescently labelled LDL taken up by the transfected HepG2 cells were determined by flow cytometry. Mean (±SD) of three experiments is shown.
Pro31–52-PCSK9 to bind to the LDLR. Moreover, in HepG2 cells transiently transfected with ΔPro31–52-PCSK9 plasmid, the amount of LDL internalized was reduced by approximately 40% compared to that of cells transfected with the WT-PCSK9 plasmid. These data showing a higher activity of ΔPro31–52-PCSK9 towards the LDLR are in agreement with previous reports [12,18,19]. Kwon et al. [12] showed that deleting residues 31–53 increased the affinity of PCSK9 to bind to the LDLR 7-fold. Benjannet et al. [18] found that deleting residues 33–58 reduced the amount of LDLRs by 60% and Holla et al. [19] observed that deleting residues 31–52 reduced the amount of LDLRs by 50%.

Fig. 4. Western blot analyses of HepG2 cells transfected with PCSK9-containing plasmids with or without residues 31–52 deleted. HepG2 cells were transiently transfected with empty plasmid, the WT-PCSK9 plasmid or various mutant PCSK9 plasmids with or without residues 31–52 deleted (Δ31–52). The amounts of PCSK9 in the media and in lysates were determined by Western blot analysis using an antibody against the C-terminal FLAG-tag. Pro-PCSK9 and mature PCSK9 (M-PCSK9) are indicated. The amounts of LDLRs in the lysates were determined by Western blot analysis using an antibody against the ligand-binding domain. β-Tubulin was used as a loading control.

Fig. 5. Internalization of furin-cleaved PCSK9 in CHO T-REx cells. CHO T-REx cells stably transfected with an LDLR plasmid were incubated with equal amounts of WT-PCSK9 (WT) or purified furin-cleaved PCSK9 (F) for 4 h, and the amounts of PCSK9 internalized were determined by Western blot analysis using an antibody against the C-terminal V5 epitope tag. Cells incubated without added PCSK9 (Ctr) in the medium were included as a control. The left panel shows the amounts of PCSK9 internalized when the cells were cultured in the absence of tetracyclin, and the right panel shows the amounts of PCSK9 internalized when the cells were cultured in the presence of tetracyclin to induce expression of the LDLR transgene. β-Tubulin was used as a loading control.

Fig. 6. Effect of furin-cleaved PCSK9 on the amount of LDLRs in HepG2 cells. HepG2 cells were cultured in medium containing 10% fetal calf serum (FCS) or in the presence of 5 mg/100 ml of lipoprotein-deficient serum (LPDS). HepG2 cells cultured in the presence of LPDS were also cultured in the absence of exogenous PCSK9 (Ctr) or in the presence of equal amounts of purified WT-PCSK9 (WT) or furin-cleaved PCSK9 (F). The amounts of LDLRs in the lysates were determined by Western blot analysis using an antibody against the ligand-binding domain. The amounts of PCSK9 internalized were determined by Western blot analysis using an antibody against the C-terminal V5 epitope tag. β-Tubulin was used as a loading control.
Our data have also shown that Pro31–52 exhibits a negative effect on the autocatalytic cleavage of PCSK9. When Pro31–52 was deleted, there was a two-fold higher relative amount of autocatalytically cleaved PCSK9 in lysates. Similar findings have been reported by Holla et al. [19] and Benjannet et al. [18]. In our study, increased autocatalytic cleavage due to deletion of Pro31–52 was not only observed for WT-PCSK9, but was observed for all PCSK9 mutants studied.

It has previously been shown that binding of PCSK9 to the LDLR in the endoplasmic reticulum promotes autocatalytic cleavage of PCSK9 [27,28]. Thus, reduced binding of PCSK9 to the LDLR in the presence of Pro31–52, could explain the inhibitory effect of this segment both on the autocatalytic cleavage and on the affinity to bind to the LDLR at the cell surface.

Because Pro31–52 also has a negative effect on the binding to the LDLR of L455X-PCSK9 which lacks the C-terminal domain, Pro31–52 does not exhibit its negative effects by inhibiting the interaction between the C-terminal domain of PCSK9 and the ligand-binding domain of the LDLR. This suggests that Pro31–52 inhibits the interaction between the catalytic domain of PCSK9 and the epidermal growth factor-like repeat A (EGF-A) of the LDLR.

It has been shown that the inhibitory effect of Pro31–52 is due to its negative charge and the requirement of a certain length [18,19]. Thus, it is reasonable to hypothesize that the mechanism for the inhibitory effect of Pro31–52 involves attraction by positively charged residues of the prodomain or the catalytic domain. This could lead to positioning of Pro31–52 so that it interferes with the binding of the catalytic domain of PCSK9 to the EGF-A repeat of the LDLR.

Irrespective of the underlying mechanism, our data and those of others indicate that the unstructured segment comprising residues 31–52 of PCSK9 represents an autoinhibitory domain. Such autoinhibitory domains in the unstructured N-terminal part of proteins represent a mechanism to regulate the function of the respective prodomain or disrupt positively charged residues of the prodomain or the catalytic domain. This could lead to positioning of Pro31–52 so that it interferes with the binding of the catalytic domain of PCSK9 to the EGF-A repeat of the LDLR.

Irrespective of the underlying mechanism, our data and those of others indicate that the unstructured segment comprising residues 31–52 of PCSK9 represents an autoinhibitory domain. Such autoinhibitory domains in the unstructured N-terminal part of proteins represent a mechanism to regulate the function of the respective proteins [29]. Typically, these autoinhibitory domains are enriched in polar and charged amino acids [30] as is the case for Pro31–52. The mechanism by which these autoinhibitory domains exist their regulatory functions, include proteolysis, post-translational modifications or interaction with proteins in trans [31].

Regarding a possible role for autoinhibition of PCSK9, one may speculate that it could serve to fine-tune the regulation of intracellular cholesterol levels by affecting the autocatalytic cleavage and thereby the exit of PCSK9 from the endoplasmic reticulum, as well as affecting the binding of secreted PCSK9 to the LDLR at the cell surface. If autoinhibition of PCSK9 does play a regulatory role in cholesterol metabolism, one may then speculate how this autoinhibition could be regulated.

Approximately 10% of proteins that are subject to autoinhibition are regulated by proteolysis [31], and proteolysis within Pro31–52 to make it shorter, would prevent autoinhibition [12,18,19]. However, as determined by cell culture experiments there are no data to indicate that proteolysis does occur within Pro31–52 to shorten this segment and thereby increase the activity towards the LDLR. One possible explanation for this, however, could be that the relevant proteolytic enzymes are not synthesized by the cell lines commonly used for in vitro experiments. Thus, proteolysis could occur in vivo by enzymes found in the extracellular fluid or in plasma.

An observation that may support the notion that proteolysis can occur within Pro31–52, is that cleavage at Arg46↓ to lead to deletion of residues 31–46, can be observed in PCSK9 purified from insect High Five cells [18]. Moreover, this shortened presequence with presequence residues 31–46 deleted, has a 4-fold increased affinity to bind to the LDLR [18], which is similar to that observed by deleting residues 31–53 [12]. This observation could suggest that there is an enzyme in High Five cells that performs the cleavage at Arg46↓. However, cleavage of the prodomain can also be observed when PCSK9 is purified from stably transfected HEK293 cells using the method described by Hollla et al. [22] (Suppl. Fig. S1). In fact, three different sizes of the prodomain were observed in samples of purified PCSK9, whereas only one prodomain fragment was observed in lysates of transfected cells (Suppl. Fig. S1). This indicates that the prodomain may be subject to post-translational modifications under certain conditions. Also, proteolysis at Arg46↓ in cell culture experiments has been observed for WT-PCSK9 and for all PCSK9 mutants studied.

If cleavage at Arg46↓ does play a part in regulating autoinhibition of PCSK9, a possible mechanism for regulating the extent of cleavage, could involve phosphorylation at Ser47. When Ser47 is phosphorylated, cleavage at Arg46↓ is prevented [32]. Also for other proteins, phosphorylated serines in the motif Arg-Ser have been shown to prevent cleavage at Arg↓ [33]. Moreover, phosphorylation has been shown to modulate autoinhibition in approximately 35% of proteins undergoing autoinhibition [31]. One may therefore speculate that the putative autoinhibition of PCSK9 is regulated in vivo by proteolysis at Arg46↓ which again is regulated by phosphorylation at Ser47.

If the positioning of the negatively charged Pro31–52 relative to the catalytic domain of PCSK9 is affected by positively charged residues of the catalytic domain, then mutations within the PCSK9 gene that create or disrupt positively charged residues of the prodomain or the catalytic domain, could affect the positioning of Pro31–52. Depending on the location of these mutant residues they may be gain-of-function or loss-of-function mutants depending on how they affect the positioning of Pro31–52. In this respect, it is striking that several gain-of-function mutants (e.g. E32K, E57K, L106R, S127R, R215H, R218S, R218H) involve basic residues.

One common feature of these gain-of-function mutants which involve basic residues, is that their LDLR-reducing activity is low as determined by in vitro studies using purified mutant PCSK9 or by transfection experiments. This is also demonstrated in our study where several of these gain-of-function mutants have an activity similar to that of WT-PCSK9, as determined by Western blot analysis or flow cytometry. Also binding studies of S127R-PCSK9 to the LDLR using plasmon surface resonance, have shown that the affinity of S127R-PCSK9 to bind to the LDLR, is similar to that of WT-PCSK9 [34,35]. Moreover, the finding that Ser127↓ is located ~40 Å from the EGF-A repeat [12] makes it unlikely that S127R-PCSK9 is a gain-of-function mutant due to increased affinity to bind to the LDLR. Furthermore, regarding mutations involving Ser127↓ the only mutant other than S127R that is a gain-of-function mutant, is S127K-PCSK9 [35] which also substitutes a neutral serine for a basic lysine. Regarding the gain-of-function mutation R215H which has been found to segregate with hypercholesterolemia [36], little or no gain-of-function activity has been found in cell culture experiments, as is shown in our study and in a previous study [36]. Thus, there is a discrepancy between the clinical phenotype in heterozygous carriers of gain-of-function mutations involving basic residues, and the results of in vitro studies to study the effect of these mutations on the amount of LDLRs. This is in contrast to the gain-of-function mutant D374Y-PCSK9 which consistently has been shown to have a great LDLR-reducing activity as determined by cell culture studies [9,34,35]. Moreover, the finding that Ser127↓ is located ~40 Å from the EGF-A repeat [12] makes it unlikely that S127R-PCSK9 is a gain-of-function mutant due to increased affinity to bind to the LDLR. Furthermore, regarding mutations involving Ser127↓ the only mutant other than S127R that is a gain-of-function mutant, is S127K-PCSK9 [35] which also substitutes a neutral serine for a basic lysine. Regarding the gain-of-function mutation R215H which has been found to segregate with hypercholesterolemia [36], little or no gain-of-function activity has been found in cell culture experiments as is shown in our study and in a previous study [36]. Thus, there is a discrepancy between the clinical phenotype in heterozygous carriers of gain-of-function mutations involving basic residues, and the results of in vitro studies to study the effect of these mutations on the amount of LDLRs. This is in contrast to the gain-of-function mutant D374Y-PCSK9 which consistently has been shown to have a great LDLR-reducing activity as determined by cell culture studies. The mechanism for D374Y-PCSK9 being a gain-of-function mutant is that a hydrogen bond is formed between Tyr374↓ of PCSK9 and His306↓ of the LDLR. This results in a 10–25-fold increased affinity of D374Y-PCSK9 to bind to the LDLR [9,34,37,38]. Thus, there appears to be two classes of gain-of-function mutations. One class consists of mutation D374Y and the other class consists of mutations affecting basic residues such as E32K, E57K, L106R, S127R, R215H, R218S, R218H. The notion that there are different mechanisms for mutations S127R and D374Y being gain-of-function mutations, has previously been suggested by Pandit et al. [35]. Our data showing that in vitro activity of WT-PCSK9 towards the LDLR and of all mutant PCSK9s tested was approximately 40% higher when Pro31–52 was deleted, and actually approached the activity of the mutant full-length D374Y-PCSK9, makes it tempting to speculate
that the mechanism for mutants other than D374Y-PCSK9 being gain-of-function mutants, involves modifications in vivo to increase their activities towards the LDLR. One may speculate that altered positioning of Pro31–52 due to gain-of-function mutations involving basic residues, could cause an increased propensity to in vivo cleavage, possibly at Arg46. An alternative hypothesis could be that altered positioning of Pro31–52 due to these basic residues, could make Pro31–52 more prone to bind to another protein found in plasma or in the extracellular fluid.

Regarding gain-of-function mutations R215H, R218H and R218S in the unstructured, conserved loop comprising residues 213–218, re-duced inactivation of the mutant PCSK9 by furin at the furin cleavage site RFHR218, could represent an alternative mechanism. Moreover, our data support those of Benjannet et al. [26] and Han et al. [39] that furin cleavage makes PCSK9 unable to bind to the LDLR and contrast those of Lipari et al. [40]. However, uncertainties still exist regarding the physiological role of furin cleavage at RFHR218.

The notion that an intact loop consisting of residues 213–218 is important for the binding of PCSK9 to the LDLR, is also supported by our data showing that introducing mutations DEDE218, makes the mutant DEDE-PCSK9 a loss-of-function mutant. This conserved loop is located close to the EGF-B repeat of the LDLR, and a monoclonal antibody recognizing this loop completely abolishes the binding of PCSK9 to the LDLR [40]. Moreover, the affinity of PCSK9 to bind to an LDL peptide consisting of both EGF-A and EGF-B, is higher than the binding of PCSK9 to a peptide consisting of EGF-A alone [37]. This could suggest that there are interactions between PCSK9 and the LDLR that cannot be identified by X-ray crystallography.

In conclusion, our data indicate that the unstructured segment comprising residues 31–52 of PCSK9 acts as an autoinhibitory domain on the activity of PCSK9 towards LDLR. We speculate that this autoinhibitory action could be regulated in vivo which may serve to fine-tune the activity of PCSK9 towards the LDLR. A possible autoinhibitory regulation of this domain may involve cleavage at Arg46 which could be regulated by the phosphorylation status of Ser47. Furthermore, altered positioning of this unstructured segment by mutant residues within PCSK9 may form the basis for the respective mutations being gain-of-function mutations due to reduced autoinhibitory activity. However, at this point these suggestions should be considered as hypotheses and further studies and investigations are needed to test these.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2016.11.003.

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[1] J.L. Goldenstein, H.H. Hobbs, M.S. Brown, Familial hypercholesterolemia, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The metabolic & molecular Basis of Inherited Disease, McGraw-Hill, New York 2001, pp. 2863–2914.

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