Interaction between the ligand-binding domain of the LDL receptor and the C-terminal domain of PCSK9 is required for PCSK9 to remain bound to the LDL receptor during endosomal acidification

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Received October 4, 2011; Revised and Accepted December 2, 2011

Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the epidermal growth factor homology domain repeat A of the low-density lipoprotein receptor (LDLR) at the cell surface and disrupts recycling of the internalized LDLR. As a consequence, the LDLR is rerouted to the lysosomes for degradation. Although PCSK9 may bind to an LDLR lacking the ligand-binding domain, at least three ligand-binding repeats of the ligand-binding domain are required for PCSK9 to reroute the LDLR to the lysosomes. In this study, we have studied the binding of PCSK9 to an LDLR with or without the ligand-binding domain at increasingly acidic conditions in order to mimic the milieu of the LDLR:PCSK9 complex as it translocates from the cell membrane to the sorting endosomes. These studies have shown that PCSK9 is rapidly released from an LDLR lacking the ligand-binding domain at pH in the range of 6.9–6.1. A similar pattern of release at acidic pH was also observed for the binding to the normal LDLR of mutant PCSK9 lacking the C-terminal domain. Together these data indicate that an interaction between the negatively charged ligand-binding domain of the LDLR and the positively charged C-terminal domain of PCSK9 is required for PCSK9 to remain bound to the LDLR during the early phase of endosomal acidification as the LDLR translocates from the cell membrane to the sorting endosome.

INTRODUCTION

The low-density lipoprotein (LDL) receptor (LDLR) is a cell-surface receptor that plays a key role in cholesterol metabolism through its ability to bind and internalize LDL (1). The LDLR is a type I transmembrane protein and the extracellular part is commonly divided into the ligand-binding domain, the epidermal growth factor (EGF) precursor homology domain and the O-linked sugar domain (2,3). The ligand-binding domain consists of seven repeats of approximately 40 amino acids each (3). Each repeat has clusters of acidic residues which coordinate one divalent calcium ion, and each repeat contains six cysteine residues involved in three disulfide bonds (4). The negative charge of the ligand-binding domain is important for ligand binding through interaction with positively charged residues of apolipoprotein-B or apolipoprotein-E on lipoproteins. The EGF precursor homology domain of approximately 400 amino acids consists of two contiguous 40 amino acid repeats, EGF-A and EGF-B, followed by a 280 amino acid β-propeller and a third 40 amino acid repeat, EGF-C (2,4). The EGF precursor homology domain is required for the conformational change and release of LDL from the LDLR at the acidic pH of sorting endosomes (5). The O-linked sugar domain of 58 amino acids is enriched in serine and threonine residues which serve as attachment sites for carbohydrates (4).

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LDL binds to the ligand-binding domain of the LDLR and is subsequently internalized by receptor-mediated endocytosis (1). At the acidic pH of the sorting endosomes, the LDLR undergoes a conformational change where the ligand-binding domain folds back on the β-propeller of the EGF precursor homology domain (6). Thus, the LDLR adopts a closed conformation in the endosomes and LDL is released from the LDLR. The released LDL is transported to the lysosomes for degradation and the LDLR is recycled back to the cell membrane (7,8).

The LDLR not only binds lipoproteins but may also bind proprotein convertase subtilisin/kexin type 9 (PCSK9) (9), which acts to reroute the LDLR to the lysosomes for degradation (10,11). PCSK9 consists of a prodomain, a catalytic domain and a C-terminal domain and undergoes autocatalytic cleavage in the endoplasmic reticulum (9). It is secreted as a complex consisting of the cleaved prodomain non-covalently bound to the mature form (9). In contrast to lipoproteins, PCSK9 does not bind to the ligand-binding domain of the LDLR. Instead, it binds to the EGF-A repeat through a patch of residues in the catalytic domain of PCSK9 (11–13). The LDLR with bound PCSK9 is internalized through clathrin-coated pits and the affinity of PCSK9 to bind to the LDLR increases at the acidic pH of endosomes (14,15). Thus, although LDL is released from the LDLR at the acidic milieu of the endosomes, PCSK9 remains bound to the LDLR and disrupts its recycling. PCSK9 therefore has an LDLR-lowering effect which acts to increase the levels of plasma LDL cholesterol (10).

The structural requirements for PCSK9-mediated degradation of the LDLR have been well characterized (16). In addition to the EGF-A repeat, at least three ligand-binding repeats and an intact β-propeller are required for the LDLR to be degraded by PCSK9 (16). However, the underlying mechanisms for these requirements are not understood. With respect to PCSK9, the catalytic domain needs to be present for PCSK9 to bind to the EGF-A repeat of the LDLR (11), and the C-terminal domain is required for bound PCSK9 to reroute the LDLR to the lysosomes (14,16–18). Recent data indicate that the role of the C-terminal domain for the activity of PCSK9 reflects its size and positive charge at acidic pH, and not the presence of specific residues involved in protein–protein interactions with complementary residues of the LDLR (18). Rather, the positively charged C-terminal domain of PCSK9 appears to be involved in electrostatic interactions with negatively charged structures of the LDLR (18).

In this study, we have performed studies to understand the mechanism behind the requirement of the ligand-binding domain of the LDLR for the LDLR:PCSK9 complex to be rerouted to the lysosomes. The strategy has been to study the binding of PCSK9 to the wild-type (WT) LDLR and to an LDLR lacking the ligand-binding domain at increasingly acidic conditions in order to mimic the transport of the LDLR:PCSK9 complex from the cell membrane to the sorting endosome.

RESULTS

Effect of PCSK9 on degradation of Δ1-7LDLR
It has previously been reported that an LDLR lacking the ligand-binding domain is able to bind and internalize PCSK9 without being degraded (16). To confirm this finding, we incubated CHO T-Rex cells stably transfected with a plasmid encoding an LDLR lacking the seven ligand-binding repeats of the ligand-binding domain (Δ1-7LDLR) with increasing concentrations of purified D374Y-PCSK9 (Fig. 1). The cells were cultured in the presence of tetracycline (1 μg/ml), in order to induce LDLR expression, for 24 h, followed by culturing in the absence of tetracycline for 8 h, before D374Y-PCSK9 was added (19). The gain-of-function mutant D374Y-PCSK9 was chosen for this experiment because it has a higher activity toward the LDLR than WT-PCSK9 (14,20). Although D374Y-PCSK9 dose-dependently reduced the amount of WT-LDLR, no reduction in the amount of Δ1-7LDLR was observed. These results therefore confirm the findings of Zhang et al. (16). The lower band of the WT-LDLR represents a degradation product that is caused by deletion of ligand-binding repeats 1–4 (21,22).

Binding of PCSK9 to Δ1-7LDLR
To study the binding of PCSK9 to Δ1-7LDLR at neutral or acidic pH, CHO T-Rex cells stably transfected with plasmids Δ1-7LDLR or WT-LDLR were incubated with 2 μg/ml D374Y-PCSK9 in media of different pH at 4°C (Fig. 2). The gain-of-function mutant D374Y-PCSK9 was chosen for this experiment in order to increase the signal-to-noise ratio. Binding of D374Y-PCSK9 to the WT-LDLR increased significantly with decreasing pH from 7.4 to 5.9 and was somewhat reduced when pH of the medium was further reduced to 5.3. A similar effect of pH was observed also for the binding of D374Y-PCSK9 to Δ1-7LDLR. Even though the amounts of the WT-LDLR and Δ1-7LDLR appeared to be similar (Fig. 2), more D374Y-PCSK9 bound to cells expressing the WT-LDLR than to cells expressing Δ1-7LDLR. Thus, even though PCSK9 is able to bind to an LDLR without the ligand-binding domain, it binds more avidly to a receptor containing the ligand-binding domain.
1-7LDLR or WT-LDLR were incubated with 10 μg/ml WT-PCSK9 for 4 h at 37°C (Fig. 3). Although the two different LDLRs were similarly expressed (Fig. 3), cells expressing WT-LDLR internalized ~8.5-fold more WT-PCSK9 than cells expressing Δ1-7LDLR. Thus, even though PCSK9 is internalized by an LDLR lacking the ligand-binding domain, the presence of the ligand-binding domain markedly increases the amount of PCSK9 internalized.

Effect of pH on the release of PCSK9 from Δ1-7LDLR

As a model to study the effect of the increasingly acidic milieu on the binding of PCSK9 to the LDLR as the LDLR:PCSK9 complex translocates from the cell membrane to the sorting endosome, CHO T-REx cells stably transfected with the plasmids Δ1-7LDLR or WT-LDLR were incubated with WT-PCSK9 at pH 5.3 for 1 h at 4°C, washed and then incubated with media of different pH for 2 h at 4°C (Fig. 4). A pH of 5.3 rather than a pH of 7.4 was chosen for this initial binding of WT-PCSK9 because the amount of WT-PCSK9 bound at pH 7.4 was too low to reliably detect reductions in the amount of bound WT-PCSK9.

WT-PCSK9 appeared to bind equally well to cells expressing each of the two receptors at pH 5.3, with no detectable release of WT-PCSK9 into the media during the 2 h incubation at pH 5.3. As the pH was increased, the majority of WT-PCSK9 bound to cells expressing the Δ1-7LDLR was released at pH ~6.1 or higher. This was in marked contrast to the amount of WT-PCSK9 released from cells expressing the WT-LDLR, where only small amounts of WT-PCSK9 were released until pH ~6.9. Thus, these data indicate that effective binding of WT-PCSK9 to the LDLR at pH 6.9–6.1 requires an intact ligand-binding domain.

Speed of the release of PCSK9 at acidic pH

If the failure of PCSK9 to degrade Δ1-7LDLR is because PCSK9 is released from Δ1-7LDLR during the first phase of endosomal acidification, this would have to occur rapidly considering that each cycle of LDLR recycling takes ~10 min (23). To study the speed of the release of PCSK9 from Δ1-7LDLR at acidic pH, CHO T-REx cells stably transfected with plasmids Δ1-7LDLR or WT-LDLR were incubated with WT-PCSK9 for 1 h at 4°C before the cells were washed with ice-cold buffer and the cells were incubated in medium without WT-PCSK9 at 4°C for 5–30 min at pH 5.3 or 6.5 (Fig. 5). As a control for the amount of WT-PCSK9 bound non-specifically to the cell membrane and to the endogenous LDLR of the CHO T-REx cells, CHO T-REx cells stably transfected with WT-LDLR plasmid cultured in the absence of tetracycline were included in the experiment. WT-PCSK9 was released from Δ1-7LDLR within 5 min at pH ~6.1 or higher. This was in marked contrast to the amount of WT-PCSK9 released from cells expressing the WT-LDLR, where only small amounts of WT-PCSK9 were released until pH ~6.9. Thus, these data indicate that effective binding of WT-PCSK9 to the LDLR at pH 6.9–6.1 requires an intact ligand-binding domain.
reduced at pH 6.5 and the amount of PCSK9 that remained bound was similar to that of cells expressing Δ1-7LDLR. Thus, in the absence of the ligand-binding domain, PCSK9 is rapidly released from the LDLR at the acidic milieu of the sorting endosome.

**Figure 4.** Release of PCSK9 from an LDLR lacking the ligand-binding domain. CHO T-REx cells stably transfected with a plasmid encoding the WT-LDLR or a plasmid encoding an LDLR lacking the ligand-binding domain (Δ1-7LDLR), cultured in the presence of tetracycline (1 μg/ml) to induce expression, were incubated with purified WT-PCSK9 (2 μg/ml) for 1 h at 4°C at pH 5.3. After washing, the cells were incubated in medium with different pH without WT-PCSK9 for 2 h at 4°C. The amount of cell-associated WT-PCSK9 and the amount of WT-PCSK9 in the media were determined by western blot analysis. Also shown are the amounts of WT-LDLR or Δ1-7LDLR as determined by western blot analysis using an antibody against the cytoplasmic domain of the LDLR which also recognizes a degradation product marked by an asterisk. A representative western blot is shown.

**Figure 5.** Speed of the release of PCSK9 from an LDLR lacking the ligand-binding domain. CHO T-REx cells stably transfected with a plasmid encoding the WT-LDLR or a plasmid encoding an LDLR lacking the ligand-binding domain (Δ1-7LDLR), cultured in the presence of tetracycline (1 μg/ml) to induce expression, were incubated with purified WT-PCSK9 (2 μg/ml) for 1 h at 4°C at pH 5.3. After washing, the cells were incubated at 4°C in medium with pH 5.3 or 6.5 without WT-PCSK9 for the indicated times. The amount of cell-associated WT-PCSK9 was determined by western blot analysis of cell lysates. Also shown is the amount of WT-PCSK9 bound to CHO T-REx cells stably transfected with a plasmid encoding the WT-LDLR cultured in the absence of tetracycline in the culture medium (background). The amounts of WT-LDLR or Δ1-7LDLR as determined by western blot analysis are shown using an antibody against the cytoplasmic domain of the LDLR which also recognizes a degradation product marked by an asterisk. A representative western blot is shown.

**Table 1.**

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**Release of PCSK9 at acidic pH from LDLRs containing two or three ligand-binding repeats**

Zhang et al. (16) have shown that an LDLR with three ligand-binding repeats is degraded by PCSK9, whereas an LDLR
with two ligand-binding repeats is not. To determine whether the underlying mechanism is due to differences in their ability to bind PCSK9 at acidic pH, we studied CHO T-REx cells stably transfected with plasmids Δ1-4LDLR or Δ1-5LDLR encoding LDLR with ligand-binding repeats 1-4 or 1-5 deleted, respectively, cultured in the presence of tetracycline (1 μg/ml) to induce expression, were incubated with purified WT-PCSK9 (2 μg/ml) for 1 h at 4°C at pH 5.3. CHO T-REx cells stably transfected with WT-LDLR plasmid or a plasmid encoding an LDLR lacking the ligand-binding domain (Δ1-7LDLR) were used as controls. After washing, the cells were incubated in medium with different pH without WT-PCSK9 for 2 h at 4°C. The amount of cell-associated WT-PCSK9 and the amount of WT-PCSK9 in the media were determined by western blot analysis. Also shown are the amounts of WT-LDLR or mutant LDLRs as determined by western blot analysis using an antibody against the cytoplasmic domain of the LDLR which also recognizes a degradation product marked by an asterisk. A representative western blot is shown.

**Figure 6.** Release of PCSK9 from an LDLR with two or three ligand-binding repeats. CHO T-REx cells stably transfected with plasmids Δ1-4LDLR or Δ1-5LDLR encoding LDLR with ligand-binding repeats 1-4 or 1-5 deleted, respectively, cultured in the presence of tetracycline (1 μg/ml) to induce expression, were incubated with purified WT-PCSK9 (2 μg/ml) for 1 h at 4°C at pH 5.3. CHO T-REx cells stably transfected with WT-LDLR plasmid or a plasmid encoding an LDLR lacking the ligand-binding domain (Δ1-7LDLR) were used as controls. After washing, the cells were incubated in medium with different pH without WT-PCSK9 for 2 h at 4°C. The amount of cell-associated WT-PCSK9 and the amount of WT-PCSK9 in the media were determined by western blot analysis. Also shown are the amounts of WT-LDLR or mutant LDLRs as determined by western blot analysis using an antibody against the cytoplasmic domain of the LDLR which also recognizes a degradation product marked by an asterisk. A representative western blot is shown.

Comparing Δ1-4LDLR and Δ1-5LDLR which contain three or two ligand-binding repeats, respectively, the Δ1-4LDLR bound more WT-PCSK9 particularly at pH 6.5 and below.

**Figure 7.** Release of L455X-PCSK9 from the LDLR. Conditioned media of HEK293 cells transiently transfected with plasmids WT-PCSK9, L455X-PCSK9 or L455X-PCSK9-DsRed were concentrated/diluted to give equal protein concentrations as determined by western blot analyses of the media using an antibody against the prodomain of PCSK9. These media were added to CHO T-REx cells stably transfected with a plasmid encoding the WT-LDLR for 1 h incubation at 4°C, pH 5.3. After washing, the cells were incubated in medium with different pH without PCSK9 for 2 h at 4°C. The amounts of WT-PCSK9, L455X-PCSK9 or L455X-PCSK9-DsRed that remained cell-associated were determined by western blot analysis of cell lysates. Also shown are the amounts of WT-LDLR of the cells as determined by western blot analysis using an antibody against the cytoplasmic domain of the LDLR which also recognizes a degradation product marked by an asterisk. A representative western blot is shown.

**DISCUSSION**

In this study, we performed experiments to determine why the ligand-binding domain of the LDLR is required for PCSK9-mediated degradation. First, we confirmed the findings of Zhang et al. (16) that an LDLR which lacks the ligand-
binding domain is able to bind and internalize PCSK9 without being degraded. The Δ1-7LDLR bound less PCSK9 than the WT-LDLR at pH 7.4 and was markedly less effective in internalizing PCSK9 than the WT-LDLR. Thus, the ligand-binding domain of the LDLR is not absolutely required for the binding and internalization of PCSK9, but its presence increases the affinity of PCSK9 to bind to the LDLR and substantially increases the amount of PCSK9 internalized.

Because recycling of Δ1-7LDLR is not disrupted by PCSK9, PCSK9 may remain bound to the Δ1-7LDLR during recycling. However, the data of Zhang et al. (16) showing that substantial amounts of PCSK9 are internalized by Δ1-7LDLR suggested that PCSK9 is released from Δ1-7LDLR in the sorting endosomes. This notion is supported by our findings that PCSK9 remained stably bound to the WT-LDLR when the pH was raised from 5.3 to 6.9, whereas PCSK9 was released from the Δ1-7LDLR within a few minutes at pH 6.1 or higher. These data therefore indicate that the negatively charged ligand-binding domain of the LDLR is required for PCSK9 to remain bound to the LDLR during the early phase of endosomal acidification as the LDLR translocates from the cell membrane (pH 7.4) to the sorting endosome (pH 6.1). Thus, in the absence of the ligand-binding domain, PCSK9 is released from the LDLR at the acidic environment of the sorting endosome in a fashion that appears to be similar to that of LDL. Both ligands will then be confined to the vesicular portion of the sorting endosomes that eventually matures to become a late endosome that fuses with a lysosome.

The data of Zhang et al. (16) suggested that the more ligand-binding repeats of the ligand-binding domain of the LDLR, the more effectively is LDLR degraded by PCSK9. Their finding that at least three ligand-binding repeats had to be present, irrespective of which, suggests that the role of the ligand-binding repeats does not reflect their involvement in specific protein–protein interactions. Rather, it appears that a certain net negative charge has to be present. Our data have shown that much more PCSK9 is released from an LDLR with two ligand-binding repeats than from an LDLR with three ligand-binding repeats at endosomal pH. Thus, together with the data of Zhang et al. (16), our data indicate that at least three ligand-binding repeats are required for PCSK9 to remain bound to the LDLR at endosomal pH. It may be inferred that the more ligand-binding repeats and the higher the negative charge of the ligand-binding domain, the stronger is the binding of PCSK9 to the LDLR at endosomal pH. The requirement for a minimum of three ligand-binding repeats for the LDLR to be degraded by PCSK9 may reflect a threshold for the net negative charge of the ligand-binding domain to interact with the positively charged C-terminal domain of PCSK9. However, it is also possible that a certain length of the ligand-binding domain is required to approach the C-terminal domain of PCSK9.

The positively charged C-terminal domain of PCSK9 must be an integrated part of PCSK9 for PCSK9 to have an activity toward the LDLR (14,16–18). This domain is characterized by an abundance of histidines which leads to an increased net positive charge at acidic pH (14,15). We have recently shown that the requirement of the C-terminal domain for the activity of PCSK9 reflects its size and positive charge and not the presence of specific residues (18). In fact, PCSK9 retained its activity toward the LDLR when the C-terminal domain had been replaced with an unrelated and similarly sized protein (18). Thus, residues of the C-terminal domain of PCSK9 do not appear to be involved in specific key-and-lock-like protein–protein interactions with residues of a matching segment in the LDLR. Rather, the positively charged C-terminal domain may be involved in an electrostatic attraction with negatively charged parts of the LDLR which is required for PCSK9 to remain bound to the LDLR at acidic conditions.

It is reasonable to assume that the negatively charged part of the LDLR involved in an electrostatic attraction with the C-terminal domain of PCSK9 is the ligand-binding domain. Our data show that L455X-PCSK9, which lacks the C-terminal domain, was released from the LDLR at mildly acidic conditions in a fashion similar to the release of WT-PCSK9 from Δ1-7LDLR. Thus, the most likely interpretation of our data is that an interaction between the ligand-binding domain of the LDLR and the C-terminal domain of PCSK9 is required for the LDLR:PCSK9 complex to remain stable during translocation from the cell membrane to the sorting endosome. The finding that L455X-PCSK9 fused to the DsRed-Express fluorescent protein restored the binding to the LDLR at acidic pH explains why this fusion protein has an LDLR-degrading activity similar to that of WT-PCSK9 (18).

Our data regarding the role of the ligand-binding domain are supported by the findings of Yamamoto et al. (24). Using a column-binding assay and conditions favoring protein binding, they found that the C-terminal domain of PCSK9 bound to the ligand-binding domain of the LDLR particularly at pH 5.4. However, such an interaction has not been detectable by methods such as immunoprecipitation (16) or studies of surface plasmon resonance (14,24). One may speculate that a weak electrostatic attraction between the C-terminal domain of PCSK9 and the ligand-binding domain of the LDLR may not be detectable by the two latter methods. Moreover, cell studies have shown that an LDLR without the EGF-A repeat is unable to bind PCSK9 (11). Thus, it appears that for PCSK9 to mediate degradation of the LDLR, the catalytic domain of PCSK9 has to bind to the EGF-A repeat of the LDLR, followed by an electrostatic interaction between the C-terminal domain of PCSK9 and the ligand-binding domain of the LDLR, which is critical during early endosomal acidification.

Our study has shown that, at pH below ~6.0, similar amounts of PCSK9 remained bound to the Δ1-7LDLR as to the WT-LDLR. Thus, the 150-fold increased affinity of PCSK9 to bind to the WT-LDLR at pH 5.4 (14,15) appears largely to be mediated by an increased affinity of the catalytic domain of PCSK9 to bind to the EGF-A repeat of the LDLR. The underlying mechanism for this seems to involve protonation of His306 in EGF-A and the formation of a salt bridge with Asp374 (25). However, additional mechanisms may also be involved as suggested by Bottomley et al. (25).

The mechanism by which an LDLR with bound PCSK9 is excluded from entering the recycling tubules of the sorting endosome and is rerouted to the lysosomes
remains to be determined. However, the underlying mechanism does not involve an interaction between adaptor proteins and the cytoplasmic domain of the LDLR, as an LDLR lacking the cytoplasmic domain is degraded by PCSK9 (26). Thus, if a third protein is involved in rerouting the LDLR:PCSK9 complex to the lysosomes, it must interact with components of the LDLR:PCSK9 complex in the lumen of the sorting endosome.

MATERIALS AND METHODS

Plasmids and transfections

The plasmid pcDNA4-LDLR encoding the full-length LDLR and the plasmid Δ1-7LDLR which encodes an LDLR without the ligand-binding domain have been previously described (18,27). The plasmid pcDNA4-LDLR was used as a template to generate plasmids Δ1-4LDLR and Δ1-5LDLR which encode LDLRs with ligand-binding repeats 1-4 or 1-5 deleted, respectively. The primer sequences used for mutageneses were: 5′-CTCGCCGCGGCGGGACTAGGGGTCTTTAC GTGTTC-3′ and 5′-GAACACGTAAAGACCCCTAGTCCC CGCCGCGGCGAG-3′ to generate Δ1-4LDLR and 5′-CT CGCCCGCGGCGGGACTAGGGGTCTTTAC GTGTTC-3′ and 5′-GAACACGTAAAGACCCCTAGTCCC CGCCGCGGCGAG-3′ to generate Δ1-5LDLR. Mutageneses were carried out using QuikChange XL Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The integrity of the plasmids was confirmed by DNA sequencing. CHO T-REx cells were stably transfected and cultured as previously described (28). The CHO T-REx cells were stably transfected with a tetracycline repressor which enables tetracycline-induced expression of genes cloned into plasmids that contain the tetracycline operator 2 element. HEK293 cells were transiently transfected with the WT-PCSK9 plasmid, L455X-PCSK9 plasmid (18) or L455X-PCSK9-DsRed plasmid (18) using Fugene HD (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. PCSK9-containing media from transfected HEK293 cells were concentrated by the use of a 30 kDa Amicon Ultra Centrifugal Filter Tube (Millipore Corp., Billerica, MA, USA) according to the manufacturer’s instructions.

Purification of PCSK9

WT or mutant PCSK9 were purified from media of stably transfected HEK293 cells as previously described (18,20). The purified PCSK9s contained a C-terminal V5-His tag.

PCSK9-mediated degradation of LDLR

The experimental conditions for determining the effect of the gain-of-function D374Y-PCSK9 mutant on the LDLRs of CHO T-REx stably transfected with WT-LDLR or mutant LDLR plasmids have been previously described (19).

Western blot analyses

Western blot analyses of cell lysates or culture media of stably transfected CHO T-REx cells were carried out as previously described (20). Briefly, cell lysates were obtained and run on 4–20% Tris–HCl Criterion Precast Gels (Bio-Rad, Hercules, CA, USA) and blotted onto Immuno-Blot PVDF Membranes (Bio-Rad). To detect PCSK9, the membranes were immunostained with a mouse anti-V5-HRP antibody (Invitrogen), a rabbit anti-His-HRP antibody (Abcam, Cambridge, UK) or a custom-made rabbit anti-PCSK9 antibody raised against residues 46–62 of the prodomain (Bethyl Laboratories, Inc., Montgomery, TX, USA). To detect the LDLR, the membranes were immunostained with a rabbit anti-LDLR antibody (EP1553Y, Abcam) which recognizes the cytoplasmic domain of the LDLR.

Cell binding, internalization and release of PCSK9 at different pH

CHO T-REx cells stably transfected with the WT-LDLR plasmid or mutant LDLR plasmids were cultured in the presence of 1 μg/ml tetracycline to induce expression of the transgenes, and in the presence of 10 μM 25-OH cholesterol (binding experiments only) to down-regulate the endogenous LDLR gene of the CHO T-REx cells. To study the binding of PCSK9 to the LDLR at the cell surface, the washed cells were resuspended in ice-cold pH buffer (pH 7.4: 50 mM Tris–HCl; pH 6.9, 6.7, 6.5, 6.3 and 6.1: 25 mM Tris–maleate; pH 5.9 and 5.3: 25 mM Tris–sulfate, all pH adjusted to 7.4) and chilled on ice for 30 min before incubation with purified D374Y-PCSK9 (2 μg/ml) for 1 h at 4°C. The cells were washed with ice-cold buffers of relevant pH and the amount of cell-associated D374Y-PCSK9 was determined by western blot analysis. To determine the amount of PCSK9 internalized, stably transfected CHO T-REx cells were incubated with WT-PCSK9 (10 μg/ml) for 4 h at 37°C and the amount of cell-associated WT-PCSK9 was determined by western blot analysis. To determine the amount of PCSK9 released from stably transfected CHO T-REx cells, the cells were first incubated with WT-PCSK9 (2 μg/ml) for 1 h at 4°C and the amount of cell-associated WT-PCSK9 was determined by western blot analysis. To determine the amount of PCSK9 released from stably transfected CHO T-REx cells, the cells were first incubated with WT-PCSK9 (2 μg/ml) for 1 h at 4°C and the amount of cell-associated WT-PCSK9 was determined by western blot analysis. To determine the amount of PCSK9 released from stably transfected CHO T-REx cells, the cells were first incubated with WT-PCSK9 (2 μg/ml) for 1 h at 4°C and the amount of cell-associated WT-PCSK9 was determined by western blot analysis.

Conflict of Interest statement. None declared.

FUNDING

This study was funded by the regular hospital budget of the Unit for Cardiac and Cardiovascular Genetics, Department of Medical Genetics, Oslo University Hospital Rikshospitalet. Thus, no external funding was received.
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