

Expression and localization of PCSK9 in rat hepatic cells

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Abstract: Proprotein convertase subtilisin/kexin type 9 (PCSK9), recently cloned in several laboratories, including ours, causes a third form of autosomal dominant hypercholesterolemia. Its mechanism of action remains unclear. We studied the expression and subcellular localization of PCSK9 in fetal and adult rat tissues associated with cholesterol homeostasis using quantitative reverse transcriptase - PCR, Western blot analysis, subcellular fractionation, and confocal immunofluorescent microscopy. PCSK9 mRNA is most abundant in yolk sac and fetal liver, but the highest expression of the protein was found in differentiated hepatoma FAO-1 cell line, which also shows the highest expression of LDLR. In FAO-1 cells PCSK9 expression is downregulated by cholesterol and 25-hydroxycholesterol and upregulated in the absence of sterols following the same pattern of expression as HMG-CoA reductase, synthase, and LDLR. Subcellular fractionation, combined with Western blotting, showed that PCSK9 is localized in the ER and intermediate vesicular compartment of the cell but not in Golgi cisternae. The mature enzyme is secreted from the liver and hepatoma cells. Double labeling with antibodies to PCSK9 and LDLR or clathrin revealed some colocalization of PCSK9 with clathrin-coated vesicles and LDLR. In conclusion, our results show that PCSK9 is processed in the ER, and the mature convertase is secreted in the plasma.

Key words: PCSK9 expression, PCSK9 localization, hepatic cells.

Résumé : La PCSK9, clonée récemment dans plusieurs laboratoires, incluant le nôtre, cause une troisième forme d'hypercholestérolémie autosomique dominante. Son mécanisme d'action demeure nébuleux. Nous avons étudié l'expression et la localisation sub-cellulaire de PCSK9 dans des tissus fœtaux et adultes associés à l'homéostasie du cholestérol, par RT-PCR quantitatif, immunobuvardage, fractionnement sub-cellulaire et immunofluorescence analysée en microscopie confocale. L'ARNm de PCSK9 était très abondant dans le sac vitellin et le foie fœtal, mais l'expression la plus forte de la protéine a été trouvée dans la lignée différenciée d'hépatome FAO-1, qui présente aussi les niveaux les plus élevés de LDLR. Dans les cellules FAO-1, l'expression de PCSK9 est régulée négativement par le cholestérol et le 25-hydroxycholestérol alors qu'elle est régulée positivement en absence de stérols, suivant ainsi le même patron d'expression que la HMG-CoA réductase, la HMG-CoA synthase et le LDLR. Un fractionnement sub-cellulaire, combiné à l'immunobuvardage a montré que PCSK9 est localisé dans le RE et les compartiments vésiculaires intermédiaires de la cellule, mais pas dans les citernes du Golgi. L'enzyme mature est sécrétée par le foie et les cellules d'hépatome. Un double marquage avec des anticorps dirigés contre PCSK9 et le LDLR ou la clathrine a révélé une co-localisation de PCSK-9 avec les vésicules recouvertes de clathrines et le LDLR. En conclusion, nos résultats montrent que PCSK9 est transformé dans le RE et que la convertase mature est sécrétée dans le plasma.

Mots clés : expression de PCSK9, localisation de PCSK9, cellules hépatiques.

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Introduction

Using suppression subtractive hybridization we previously

identified cDNA clones overexpressed in 13 d fetal compared with adult liver. One of these clones (2G8) was found to encode a novel putative serine protease–subtilase (Petkov

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Abbreviations: ADH, autosomal dominant hypercholesterolemia; APOB, apolipoprotein B100; ED, embryonic day; ER, endoplasmic reticulum; ERGIC, ER/Golgi intermediate compartment; H89, isoquinolinesulfonamide; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HMGCS, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; IDL, intermediate-density lipoprotein; IF, immunofluorescence; IP, immunoprecipitation; LDL, low-density lipoprotein; LDLR, LDL receptor; NARC-1, neuronal apoptosis related convertase1; 25-OH, 25-hydroxycholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; PVDF, polyvinylidene fluoride; RT, reverse transcriptase; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element binding protein; VLDL, very low-density lipoprotein.

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et al. 2000). The rat, human, and mouse cDNAs of this serine protease were cloned by Millennium Pharmaceuticals (Cambridge, Mass.) and by the Mammalian Gene Collection Team and given the name NARC-1 and PCSK9 (Chiang 2001; Strausberg et al. 2002). PCSK9/NARC-1 is a secretory K-like subtilase with a signal sequence for release from the ER (Seidah et al. 2003) with unknown function. The enzyme undergoes autocatalytic intramolecular processing, resulting in the cleavage of its prosegment and the generation of an active proteinase (Seidah et al. 2003; Naureckiene et al. 2003).

Mutations in PCSK9 were found to be the cause of a third form of ADH (Abifadel et al. 2003; Timms et al. 2004; Leren 2004; Shioji et al. 2004). Increased production of APOB, VLDL, IDL, and LDL was shown in human subjects carrying a mutation in PCSK9 (Ouguerram et al. 2004). Using Affymetrix microarrays, Horton and colleagues (2003) found that NARC-1/PCSK9 was upregulated in SREBP-1a and SREBP-2 transgenics and downregulated in SCAP-deficient mice. In a similar study PCSK9 was also identified among downregulated genes in the livers of mice subjected to a high cholesterol diet and upregulated in transgenic mice overexpressing truncated SREBP-1a and SREBP-2 (Maxwell et al. 2003).

It has been shown that the expression of PCSK9 mRNA was induced in cholesterol-depleted and statin-treated cells (Dubuc et al. 2004). Adenoviral-mediated overexpression of PCSK9 in mice caused an increase in plasma LDL-cholesterol and depletion of the LDL receptor from the liver, suggesting that PCSK9 regulates LDLR expression and the LDL-cholesterol level by a novel mechanism (Maxwell and Breslow 2004; Benjannet et al. 2004; Park et al. 2004). How is this regulation achieved? Is it through increased turnover of the receptor (Maxwell et al. 2005) or its retention in the ER (Park et al. 2004), through its degradation in the post-endoplasmic reticulum (Maxwell et al. 2005) or some other mechanism? Whether LDLR depletion is the only cause of elevated LDL cholesterol (Maxwell and Breslow 2004; Park et al. 2004) or whether PCSK9 also affects APOB production and lipoprotein secretion (Benjannet et al. 2004) remain open questions. Nothing is known about the function of PCSK9 in fetal liver or whether it is related only to cholesterol homeostasis.

To understand the function of PCSK9 in fetal rat liver, we searched for a cell line in which the expression of this gene is regulated. We found that the differentiated rat hepatoma FAO-1 cell line expresses PCSK9, and its expression is regulated by cholesterol. Using custom made antibodies against this enzyme and immunofluorescent techniques, we found that it resides in the ER and ER/Golgi intermediate compartments and that it is secreted in the rat plasma.

Material and methods

Animals, blood and serum collection

Pregnant female and normal male Sprague–Dawley rats were purchased from Taconic Farms (German Town, N.Y.). All studies with animals were conducted under the protocol approved by the Animal Care Institute of the Albert Einstein College of Medicine.

Antibodies

Rabbit polyclonal antibodies specific for rat PCSK9 were raised against 3 polypeptides: (i) close to the N-terminus (ATATFRRCCKGA; N-Ab), which detects only the proenzyme; (ii) internal peptide (CKALNAFGGEGVYAVAR; I-Ab); and (iii) close to the C-terminus (CRSRPSAKAS; C-Ab). I-Ab and C-Ab recognize both forms of the enzyme. The antibodies were produced and affinity-purified by Covance (Denver, Penn.). The primary antibodies used are listed in Table 1. Secondary antibodies for IF microscopy made in donkeys were purchased from Jackson ImmunoResearch Laboratories (West Grove, Penn.) and used at a dilution of 1:100. Horseradish peroxidase (HRP)-conjugated anti-chicken IgG antibody was from Pierce Biotechnology Inc. (Rockford, Ill.) and used at a dilution of 1:50 000. All other secondary antibodies for Western blots were purchased from Amersham Biosciences (Piscataway, N.J.) and used at the same dilution.

Cloning of PCSK9

The clone 2G8, previously identified by us, comprised 240 bp (Petkov et al. 2000). The full length cDNA of this clone was obtained from fetal liver RNA using GeneRacer Kit (Invitrogen Corp., Carlsbad, Calif.), primers specific for the original clone, and RNA oligonucleotides (oligos) included in the GeneRacer Kit, following the procedure recommended by the supplier. For the transfection experiments Pcsk9 cDNA was cloned into pcDNA3.1 B (Invitrogen) as a fusion protein with c-myc tag and the obtained construct named pcDNA/Pcsk9/c-myc.

Cells, cell lines, and maintenance of the cells

Fetal hepatoblasts were isolated from ED14–ED17 fetuses and cultured as described previously (Dabeva et al. 2000; Oertel et al. 2003).

Human hepatoma HepG2 (HB-8065), rat hepatoma Reuber H-35 (CRL-1600), and SV-40 transformed simian fibroblasts COS-7 (CRL-1651) were purchased from the American Type Culture Collection (Manassas, Va.). The differentiated hepatoma cell line FAO-1 was described previously (Deschatrette and Weiss 1974). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen Corp., Grand Island, N.Y.) containing 10% fetal calf serum (FCS), 100 U penicillin/mL, and 100 µg streptomycin/mL.

For experiments with cholesterol, FAO-1 and HepG2 cells were plated in 6-well dishes at a concentration of $2.5\text{--}3.0 \times 10^5$ cells per well in DMEM medium supplemented with 10% FCS. Twenty four h later the cells were washed, 2 of the wells received regular medium (control), and the other 4 received DMEM supplemented with 10% lipoprotein-deficient bovine calf serum (Biomedical Technologies Inc., Stoughton, Mass.). Loading of the cells (2 wells) with cholesterol was done 24 h later by adding 50 µg of cholesterol/mL and 5 µg of 25-OH/mL (both from Sigma-Aldrich, St. Louis, Mo.) in 0.05% ethanol (final concentration). After 24 h RNA and protein were isolated as described below.

For brefeldin A and H89 (Sigma-Aldrich) treatment FAO-1 cells were grown on cover slips and treated with 10 µg of brefeldin A/mL for 40 min alone or with 10 µg of brefeldin A/mL followed by 10 min of incubation with 100 µmol of H89/L or with H89 alone for 30 min.

Table 1. Antibodies used in this study.

Antibody (clone name)	Antigen (species)	Working dilution		Company	Isotype	Catalog No.
		Western blot	IF			
c-myc (clone 9B11)	410-419* (human)	1:1000	—	Stressgene (San Diego, Calif.)	Mouse IgG2a	2276
LDL receptor	29-205* (human)	1:5000	1:250	Abcam (Cambridge, Mass.)	Chicken IgY	ab14056
Clathrin heavy chain (clone 23)	4-171* (rat)	1:1000	1:250	BD biosciences (San Diego, Calif.)	Mouse IgG1	610499
Rab4 (clone 7)	97-213* (human)	1:1000	—	BD biosciences (San Diego, Calif.)	Mouse IgG1	610888
Rab7 (clone H-50)	158-207* (human)	1:1000	—	Santa Cruz (Santa Cruz, Calif.)	Rabbit IgG	sc-10767
Protein disulfite isomerase	499-509* (rat)	1:1000	1:200	Sterssgen (San Diego, Calif.)	Mouse IgG1	SPA-891
Golgi/58K (clone 9)	whole protein (rat)	1:1000	1:50	Sigma-Aldrich (St. Louis, Mo.)	Mouse IgG1	G 2404
GM130 (clone 35)	869-962* (rat)	1:250	—	BD biosciences (San Diego, Calif.)	Mouse IgG1	610823
COPII	1-18* (rat)	1:1000	1:200	Affinity BioReagents (Golden, Colo.)	Rabbit IgG	PA1-069
Alpha-fetoprotein	whole protein (rat)	—	1:200	Gift from Dr. S. Sell	Goat IgG	—

*Amino acids.

Isolation of RNA, RT-PCR, and quantitative RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen). RT reaction was carried out with Super Script II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Rat-specific primers were chosen with the Primer3 program (Rozen and Skaletsky 2000). National Centre for Biotechnology Information gene accession numbers, primer sequences, and amplicon sizes are given in Table 2.

Real-time PCR reaction was carried out with SYBR Green PCR Master Mix on ABI Prism sequence detection system 7000 (Applied Biosystems, Warrington, UK). The relative ratio and standard deviation were calculated using comparative C_T method ($\Delta\Delta C_T$ value) as recommended by the manufacturer. The data were normalized to the expression level of glyceraldehydes-3 phosphate dehydrogenase.

Protein isolation, immunoprecipitation and Western blots

Cells were lysed with 1 mL of IP lysis buffer: in mmol/L: 20 Hepes (pH 7.4), 2 EGTA, 2 EDTA, 150 NaCl, 1% Triton X-100, 5% glycerol supplemented with protease inhibitors Complete Mini (Roche Diagnostics, Branchburg, N.J.), and 1 phenylmethanesulfonyl fluoride (PMSF) (Sigma). Cell lysates were centrifuged at 10 000g for 10 min, and the amount of protein was measured with a bicinchoninic acid kit (Pierce).

IP experiments were performed with Seize Primary Mammalian Immunoprecipitation Kit (Pierce). The antibodies to Pcsk9 and c-myc were immobilized on the beads and processed according to the supplier's manual. The immunoprecipitated protein was washed and boiled in loading buffer (Bio-Rad, Hercules, Calif.) with 5% 2-mercaptoethanol (Sigma) before loading.

For determination of PCSK9 in the plasma, blood was drawn in the presence of EDTA, PMSF was added to a final concentration of 1 mmol/L, and the blood was centrifuged at low speed for 30 min at 4 °C. Plasma was filtered through a 45 µm filter, diluted 10 fold with 0.15 mol NaCl·L⁻¹ / 50 mmol Tris-HCl·L⁻¹, pH 7.4 / 1% Triton X-100 and subjected to immunoprecipitation overnight. Commercial available rat serum from Sigma (cat. No. R9759) was also used as a control.

For Western blotting 20–50 µg of total cell protein was resolved in 7.5% or 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk and incubated consecutively with primary and secondary antibodies, and the signal from HRP was developed with Super Signal West Femto Maximum Sensitive Substrate (Pierce). The chemiluminescence was visualized on Kodak X-Omat or Fuji Super RX films (Fisher Scientific, Pittsburgh, Penn.).

Immunofluorescent microscopy

FAO-1 cells or hepatoblasts, grown on cover slips, were fixed in either acetone or 4% paraformaldehyde for 10 min at 4 °C, permeabilized with 0.1% Triton X-100, treated with sodium borohydrate, and blocked with 5% donkey serum / 1% BSA. The primary antibodies were applied for 1 h at room temperature and the secondary, fluorescent conjugated antibodies for 40 min. Pictures were taken with a Leica AOBs confocal microscope or Olympus IX70 inverted microscope.

Separation of the subcellular fractions and cell surface protein biotinylation

Different cellular fractions were obtained by linear sucrose gradient as described elsewhere (Bostrom et al. 1986). Briefly, eight 75 mm flasks with 80%–90% confluent FAO-1 cells were washed, scraped in PBS, washed in 0.25 mol sucrose·L⁻¹ / 3 mmol imidazol·L⁻¹, pH 7.4, and broken in a Dounce homogenizer at 0 °C. The postnuclear supernatant obtained after 15 min of centrifugation at 1300g was adjusted to 520 µL with 0.25 mol sucrose·L⁻¹ / 3 mmol imidazol·L⁻¹, pH 7.4, and layered on top of a 3 mL sucrose gradient (22%–50% w/w) / 3 mmol imidazol·L⁻¹, pH 7.4, over a 0.48 mL cushion of 50% (w/w) sucrose. After centrifugation for 15 h at 33 400 rpm at 4 °C in a Beckman SW 60 rotor, 28 fractions of 140 µL each were collected from the bottom of the tubes. The fractions were supplemented with sample buffer and 5% 2-mercapto ethanol, and boiled before loading.

Cell surface proteins were biotinylated with EZ-link sulfo-

Table 2. Sequence of primers used in real-time RT-PCR with SYBR green.

mRNA	Accession No.	Forward primer (5'→3')	Reverse Primer (5'→3')	Amplicon size (bp)
PCSK9	AY847775	gcactggagaaccacacagg	tggtgcatgacattgcttc	101
SREBF2	XM_216989	cacctgtggagcagctctaa	tgccagagtgtgtcctcag	104
LDLR	NM_175762	cctcagctaccagcacctct	agccatgtcacttggaatt	97
HMGCs1	NM_017268	tcgaagtatcgagagttctgt	gcctggcaatggactctg	92
HMGCR	NM_013134	agttgcgcctctgtgactt	ccagcatcaacactcaatgc	93
GAPDH	NM_017008	ggcattgctctcaatgacaa	atgtaggccatgaggtccac	94

NHS-biotin reagents (Pierce). Briefly, 80%–90% confluent FAO-1 cells were washed and incubated with 10 mmol EZ-link sulfo-NHS-biotin reagents/L for 30 min at 4 °C. After completion of the reaction, cells were washed with PBS (pH 7.0) / 100 mmol glycine·L⁻¹. Biotinylated proteins were recovered with avidin-linked agarose beads (Pierce). One-third to one-half of the biotinylated proteins and 50 µg of nonbiotinylated proteins were fractionated on SDS-PAGE.

Results

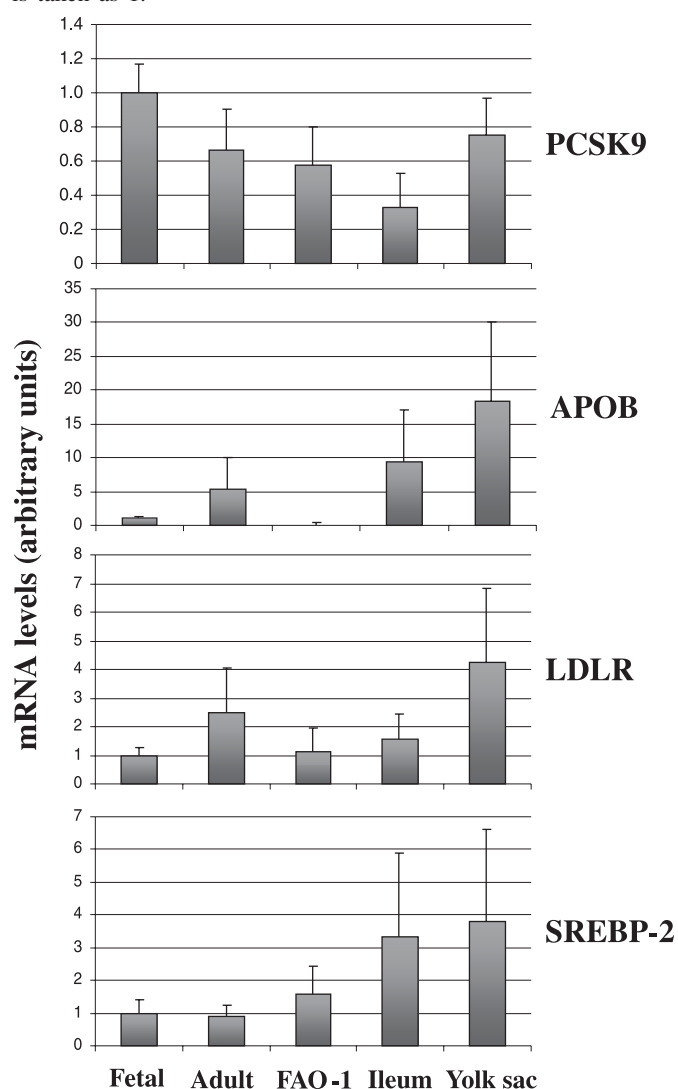
Rat PCSK9 cDNA

The full-length rat PCSK9 cDNA was cloned as described in Materials and methods, sequenced, and deposited in GenBank under accession number AY847775 (protein accession No. AAW31850). PCSK9 cDNA is 3350 nucleotides in length and codes for a protein of 691 amino acids. Two nucleotide substitutions between our clone and the previous sequence deposited in GenBank (AX207690 and NM_199253) were identified: at position 69 T instead of A and at position 71 T instead of C, which results in a protein that is 55 amino acids shorter than that given in AX207690 and NM_199253. The correctness of these nucleotides was confirmed by sequencing genomic DNA from Sprague–Dawley rats. The N-terminal sequence of the protein reported by us (deduced from nucleotide sequence) is consistent with the beginning of human and mouse PCSK9. Rat PCSK9 has the same length as the mouse protein.

Expression of PCSK9 mRNA in rat hepatic cells, fetal liver, and yolk sac

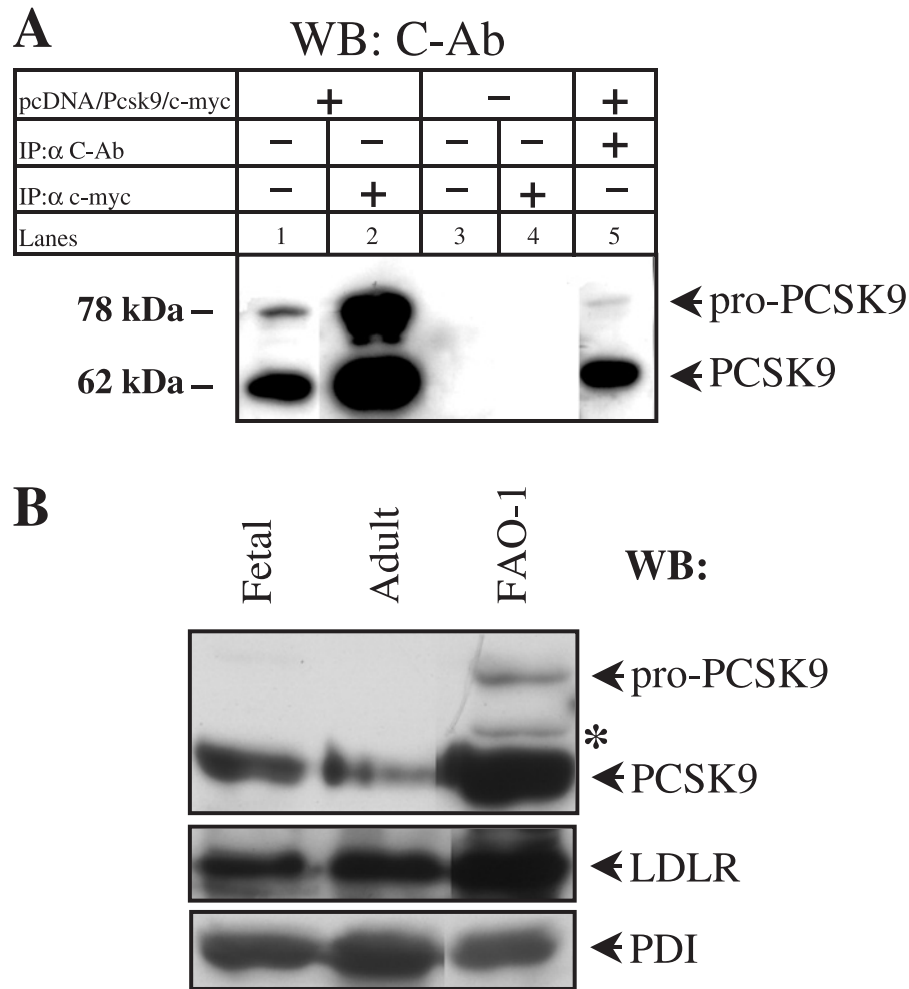
The expression of PCSK9 mRNA was studied in several tissues that maintain cholesterol homeostasis during embryogenesis and in adult rats: fetal and adult liver, yolk sac, and intestine. We also analyzed its expression level in the differentiated rat hepatoma cell line FAO-1, which could be a good in vitro model for studying PCSK9 function. The highest expression of PCSK9 mRNA was observed in fetal liver and yolk sac, and the lowest in the intestine (Fig. 1). The highest expression of APOB was also found in the yolk sac, the principal producer of LDL during early and mid-fetal life (Terasawa et al. 1999; Farese et al. 1996). In contrast, ApoB mRNA expression in the fetal liver was barely detectable compared with that of the other 2 tissues: adult liver and intestine. The same reverse pattern of expression was found for SREBP-2, the cholesterol-dependent activator of transcription of PCSK9. The abundance of LDLR mRNA in adult liver and yolk sac was much higher than that in fetal

Fig. 1. Expression of PCSK9 mRNA in rat fetal and adult tissues. Quantitative RT-PCR for PCSK9, APOB, LDLR, and SREBP-2 mRNAs in ED 14 fetal liver (Fetal), adult liver (Adult), FAO-1 cell line (FAO-1), ileum, and yolk sac. mRNA levels were normalized to GAPDH mRNA. mRNA abundance in fetal liver is taken as 1.



liver, indicating high uptake of exogenous cholesterol by these organs. These data suggest that high expression level of PCSK9 in fetal liver does not correlate with high production of LDL or LDLR, and that the expression in fetal liver may not be dependent merely on SREBP-2.

Fig. 2. (A) Specificity of PCSK9 C-Ab. Cos-7 cells were transfected with pcDNA (lanes 3 and 4) or with rat pcDNA/Pcsk9/c-myc (lanes 1, 2 and 5). Total cell lysate (lanes 1 and 3) and IP were carried out with C-Ab (lane 5) or c-myc antibodies (lanes 2 and 4), fractionated on SDS-PAGE, and blotted as described in Materials and methods. The PVDF membrane was probed with C-Ab antibodies. Molecular mass of processed (PCSK9) and unprocessed PCSK9 (pro-PCSK9) is given in kDa on the left. (B) Expression of PCSK9 in fetal, adult liver, and differentiated hepatoma FAO-1 cell line. Total cell lysates of fetal, adult liver, and FAO-1 cell line were fractionated on SDS/PAGE and blotted on PVDF membrane, and the convertase (PCSK9), LDLR, and PDI (protein disulfide isomerase) were detected with C-Ab, chicken anti-LDLR antibody, and rabbit anti-PDI, respectively. PDI is used as loading control. *, unspecific band recognized by anti-PCSK9 (C-Ab) antibodies.



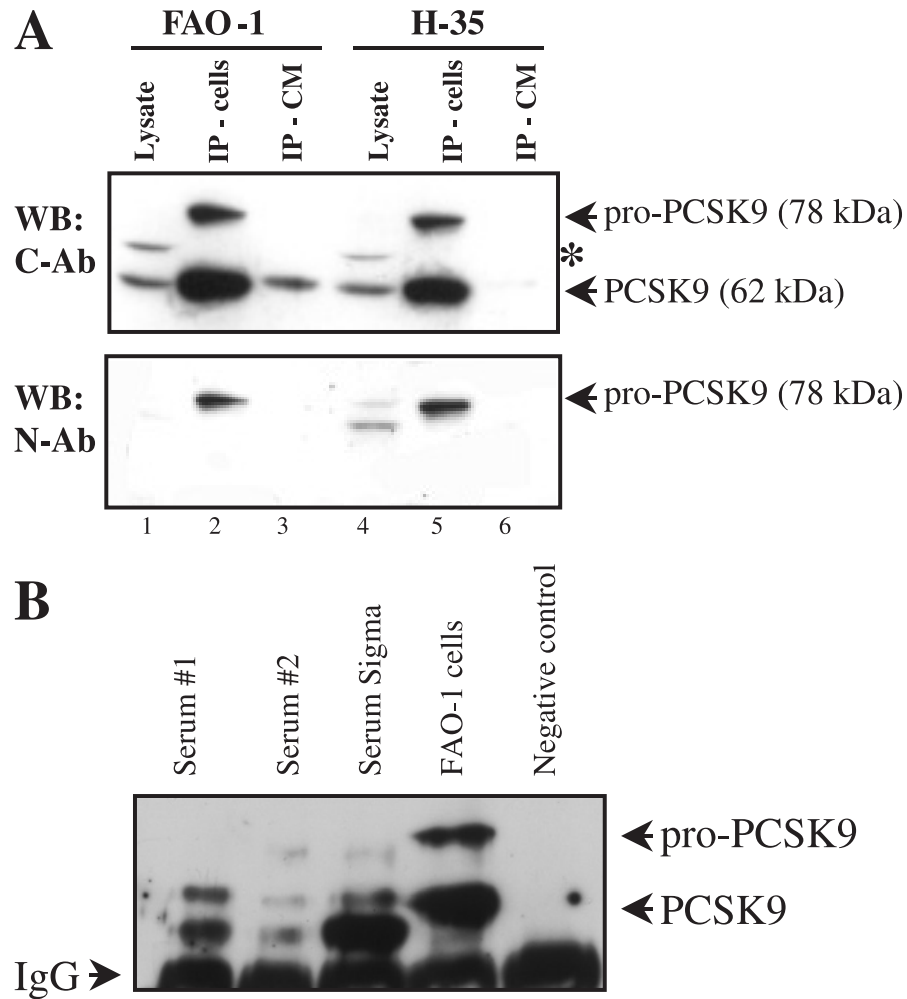
Immunochemical detection of rat PCSK9

To study the expression of PCSK9 in liver cells, we prepared custom antibodies as described in Materials and methods. These antibodies were tested on Western blots from total cell lysates or after IP and in IF experiments. In order to assess their specificity, we transiently transfected COS-7 cells with rat PCSK9 cDNA clone, tagged with the c-myc epitope (pcDNA/PCSK9/c-myc). Whole cell lysate or immunoprecipitated lysate with anti-c-myc antibody proteins from mock transfected cells did not react with C-Ab (Fig. 2A, lanes 3 and 4), showing that this antibody is specific for rat PCSK9. Total protein lysates and IP proteins with anti-c-myc from COS-7 cells transfected with pcDNA/PCSK9/c-myc reacted with the C-Ab. A light band of approximately 78 kDa and a strong band, corresponding to 62 kDa, revealed the 2 forms of rat PCSK9: the proconvertase (pro-PCSK9) and mature convertase (PCSK9) (Fig. 2A lanes 1 and 2). When

IP was carried out with C-Ab, the Western blot of precipitated proteins displayed the same 2 forms of PCSK9 (Fig. 2A, lane 5). Re-probing the membrane with N-Ab revealed only the upper light band with molecular mass of 78 kDa (data not shown). These results proved the specificity of our custom-made antibodies.

The abundance of rat PCSK9 was determined in fetal and adult rat liver and FAO-1 cells, which expressed high levels of PCSK9 mRNA. Western blots of total cell lysates from fetal liver revealed a high level of processed convertase and a very small amount of proconvertase. In adult liver, expression of PCSK9 compared with fetal liver was low, and the proconvertase was not detectable. On the other hand, the expression of pro- and processed convertases was very high in FAO-1 cells (Fig. 2B). In contrast, the expression of LDLR in fetal liver was much lower than in adult liver; highest expression was observed in FAO-1 cells (Fig. 2B). The sub-

Fig. 3. (A) Expression of PCSK9 in FAO-1 and Reuber H-35 cell lines. Total cell lysate (lysate), and immunoprecipitated protein with C-Ab from total cell lysate (IP-cells) and from the medium (IP-M) were fractionated and blotted to PVDF membrane. The same membrane was consequently probed with C-Ab (WB: C-Ab), stripped, and then re-probed with N-Ab (WB: N-Ab). *, nonspecific band recognized by anti-PCSK9 (C-Ab) antibodies. (B) Immunoprecipitation of serum. A half millilitre of serum from 2 different animals (Serum nos. 1 and 2) and regular serum from Sigma (Serum Sigma) was immunoprecipitated as described in Materials and methods. Cell lysate from FAO-1 cells (FAO-1) was used as a positive control. Membrane was probed consequently with C-Ab and I-Ab. Western blot with I-Ab is shown. Pro-PCSK9 (pro-PCSK9), mature forms of the PCSK9 (PCSK9), and IgG heavy chain (IgG) are designated.



stantial difference between mRNA and protein expression in the 3 different hepatic tissues implies that PCSK9 could be post-translationally regulated.

Secretion of PCSK9 from hepatic cells

It was reported previously that PCSK9 is secreted from HepG2 cells (Seidah et al. 2003; Naureckiene et al. 2003; Benjannet et al. 2004). To determine whether secretion of PCSK9 from hepatic cells is of physiological importance we tested 2 cell lines: Reuber H-35 and FAO-1 cells. Secreted PCSK9 was detected in the medium after IP with C-Ab. In the control lanes (2 and 5, Fig. 3A) after IP with C-Ab and visualization with either C-Ab or N-Ab, 2 bands corresponding to the proenzyme and mature enzyme were detected. The amount of secreted protein from FAO-1 cells was clearly detectable, while that secreted from Reuber H-35 was negligible (Fig. 3A, upper panel). Immunodetection with N-Ab did not produce a band corresponding to the proconvertase (Fig. 3A, lower panel). These results show that the secretion

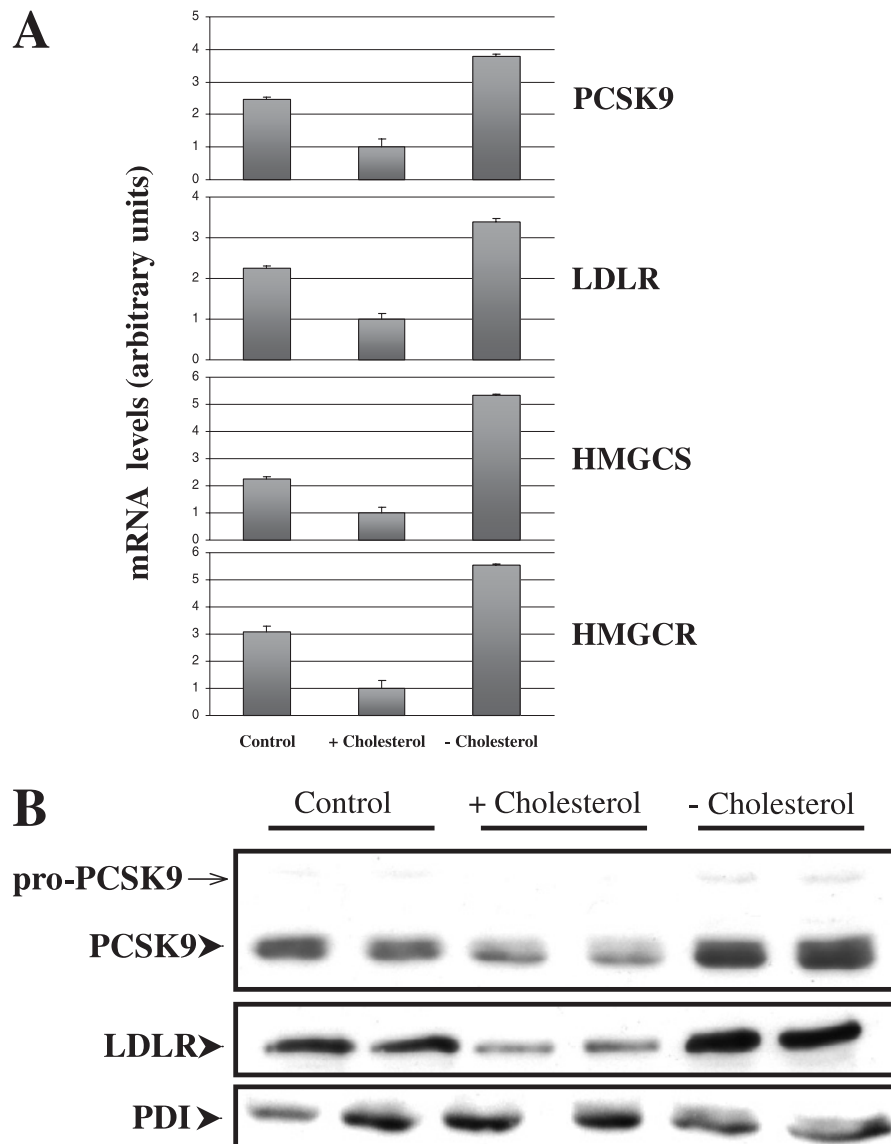
of PCSK9 from different hepatoma cell lines differs and that only the mature convertase is secreted from the cells.

IP of PCSK9 from normal rat plasma and serum revealed that its secretion from the liver is a normal process (Fig. 3B). These results were obtained with the plasma from several animals and also with normal rat serum (Sigma). Persistently, only the mature form of PCSK9 was found when a third antibody against the mature form of the PCSK9 (I-Ab) was used (Fig. 3B).

Regulation of PCSK9 expression by cholesterol

To prove directly that the expression of PCSK9 is dependent on cellular cholesterol, FAO-1 cells and HepG2 cells (not shown) were grown in 3 different media: cholesterol depleted, cholesterol/25-OH loaded, and control medium. In both cells lines, cholesterol depletion caused significant upregulation of mRNA for PCSK9 and also for HMGCR, HMGCS, and LDLR. Conversely, loading the cells with cholesterol/25-OH led to a significant reduction of mRNA abundance for all 3

Fig. 4. Regulation of PCSK9 expression in FAO-1 cell line by cholesterol. (A) Quantitation of mRNA was performed with Q-RT/PCR as described in Materials and methods, in cholesterol supplemented (+cholesterol), cholesterol depleted (–cholesterol), and untreated (control) FAO-1 cells. mRNA level of PCSK9, LDLR, HMGCS, and HMGCR are given in arbitrary units. (B) Changes in PCSK9 protein and LDLR in untreated (control), cholesterol supplemented (+cholesterol), and cholesterol depleted (–cholesterol) FAO-1 cells. Protein disulfide isomerase (PDI) is used as loading control. The results from 2 independent experiments are presented.



enzymes and LDLR (Fig. 4A). The changes in PCSK9 and LDLR expression followed the changes in mRNA expression for these proteins (Fig. 4B). These data demonstrate directly that PCSK9 is involved in maintenance of cellular cholesterol homeostasis and that FAO-1 cells are a good rat hepatoma model for studying regulation of PCSK9 expression.

Localization of PCSK9 revealed by IF

The cellular distribution of PCSK9 in FAO-1 cells was analyzed by double IF confocal microscopy (see Materials and methods). PCSK9 was detected with C-Ab or N-Ab. Cis-Golgi cisternae were revealed with monoclonal antibody against p58K protein. The results presented in Fig. 5A show a narrow distribution of the proconvertase (N-Ab), delineating the nucleus, reminiscent with its ER localization and more

diffuse vesicular distribution of the mature form (C-Ab). This suggests that PCSK9 may be also located in transport vesicles budding from the ER. Very light staining for mature PCSK9 was detected in cis-Golgi, which could be attributed to background staining; the proprotein was not detected (Fig. 5A).

These data strongly suggest that (i) the proconvertase is in the ER, where it is processed and (ii) mature PCSK9 is not localized in the Golgi but, rather, in the ER and possibly in transport vesicles. In primary culture of ED 14 hepatoblasts, alpha-fetoprotein was abundant in the Golgi cisternae, but PCSK9 was again absent from Golgi (Fig. 5B). Further experiments on PCSK9 subcellular distribution were performed using anti-clathrin heavy chain and anti-LDLR antibodies in colabeling experiments. We could expect colocalization of

clathrin and PCSK9 if the enzyme was secreted and taken by endocytosis in early endosomes (Fig. 5C). Double labeling experiments revealed that some of the convertase was colocalized with clathrin. Double immunolabeling of PCSK9 and LDLR also showed some vesicular colocalization of the 2 proteins. The nature of these vesicles will be the subject of future analyses.

Subcellular localization of PCSK9 revealed by gradient centrifugation

To determine whether PCSK9 is localized only in the ER or is also present in transport vesicles in the ERGIC of the cells, we performed subcellular fractionation of FAO-1 cells using discontinued sucrose gradients (see Materials and methods). The individual fractions were subjected to Western blot analysis with antibodies to PCSK9 and antibodies specific for cell organelles (Fig. 6A). Although PCSK9 showed a broad distribution throughout the gradient, it was not present in the preparation of pure nuclei or in the cytosol (data not shown). The proconvertase was present only in fraction 10–15 corresponding to the rough ER (Bostrom et al. 1986), revealing the site of its synthesis and processing. Electron microscopy analysis of these fractions revealed that they are composed exclusively of rough ER (data not shown). Most of the mature form was also present in this compartment (fraction 10–15). The rest of the convertase was distributed in fraction 16 through 21. It should be noted that the fractions containing the largest amount of PCSK9 were also rich in coatomer protein complex II (COPII) vesicles (fraction 11–14 and 17–23), showing that PCSK9 is also localized in the ERGIC. The light PCSK9 fractions (fraction 20–21) also overlapped with LDLR and Rab4 and partially with clathrin, suggesting that there could be small amounts of PCSK9 in early endosomes. In accordance with the immunofluorescent study above, PCSK9 did not cosediment with the Golgi apparatus (GM130 or p58K) or with late endosomes (Rab7).

To determine whether some of the convertase is localized on the plasma membrane, we labeled cell surface proteins with biotin and isolated the biotin-labeled fraction with avidin beads (see Materials and methods). As seen in Fig. 6B, a small amount of mature PCSK9 could be detected on the plasma membrane of FAO-1 cells. It has to be noted, however, that this amount is very small compared with that of total cell lysates. As could be expected, the glycosylated form of LDLR was also present on the cell membrane (Fig. 6B).

Localization of PCSK9 after treatment with brefeldin A and H89

To obtain independent proof that PCSK9 is localized in ERGIC, we treated FAO-1 cells with brefeldin A and then incubated them with H89 or H89 alone. PCSK9 was revealed with C-Ab, cis-Golgi with p58K antibody, and COPII with antibody produced in rabbit (data not shown). Brefeldin A is known to inhibit ARF1-dependent coat protein binding to Golgi membranes (Donaldson et al. 1992; Jackson and Casanova 2000), preventing formation and transport of COPI and clathrin-coated vesicles, which results in fusion of the cisternae with ER (Scales et al. 2000; Kreis et al. 1995). While COPII vesicles are not initially affected, addition of a second drug, isoquinoline H89, which inhibits the small GTPase Sar1, prevents COPII polymerization and blocks ER

to Golgi vesicle transport, causing immediate collapse of Golgi (Aridor and Balch 2000; Lee and Linstedt 2000). When brefeldin A was added to FAO-1 cells, there were no noticeable differences in the distribution of PCSK9 between treated and untreated cells (Fig. 7). Brefeldin A treatment followed by 10 min of incubation with H89 caused collapse of Golgi body and reduction of PCSK9 (Fig. 7). The same changes occurred when the cells were treated for 30 min with H89 alone: the label for PCSK9 was confined to the ER (Fig. 7). These data clearly showed that PCSK9 is localized in the ERGIC of the cells.

Discussion

This work describes our initial studies on a novel convertase, PCSK9, and provides evidence regarding its subcellular localization and secretion from hepatic tissues and cells. We have shown that PCSK9 is not only expressed in fetal and adult rat liver but is also highly expressed in FAO-1 cells and in the yolk sac of rat fetuses. While these studies were in progress, Dubuc and colleagues (2004) reported that PCSK9 mRNA expression in HepG2 cells is regulated by cholesterol. Our work extends this conclusion for the differentiated rat hepatoma cell line FAO-1, for which we showed that PCSK9 together with other cholesterol dependent enzymes, HMGCR, HMGCS, and LDLR, is down-regulated upon addition of cholesterol and upregulated in cholesterol-depleted medium. We conclude that this differentiated hepatoma cell line is a good model for studying endogenous PCSK9 expression and PCSK9-dependent cholesterol homeostasis.

Our results show further that there is a reverse relation between PCSK9 and LDLR expression in adult and fetal liver, which suggests that the high level of convertase in fetal liver could maintain the low level of LDLR (Fig. 2B). However, such a relation could not be observed for FAO-1 cells, in which both proteins, PCSK9 and LDLR, are highly expressed, indicating that this convertase may have additional function in rapidly growing cells.

It is noteworthy that organs with high production of APOB highly express PCSK9. In early and mid-gestation in mice the yolk sac is a major producer of APOB, VLDL, and LDL; towards the end of fetal life, the liver gradually becomes the principal organ of lipoprotein synthesis and secretion (Terasawa et al. 1999; Farese et al. 1996). Here we showed that PCSK9 is also highly expressed in the yolk sac. In adult animals, the highest expression of PCSK9 is found in the liver and then in the intestine (Seidah et al. 2003) (Fig. 1). These results suggest that there is a direct link between PCSK9 expression and APOB production. Such an assumption does not disagree with the data of other investigators showing that overexpression of PCSK9 causes downregulation of LDLR in mouse liver and HepG2 cells (Maxwell and Breslow 2004; Benjannet et al. 2004; Park et al. 2004; Maxwell et al. 2005). It has been reported that LDLR mediates APOB degradation in the pre-secretory pathway (Twisk et al. 2000; Gillian-Daniel et al. 2002; Larsson et al. 2004). We can hypothesize that PCSK9 (or its putative substrate) is the factor that triggers release of APOB from LDLR and subsequent degradation of the receptor. If this were the case, in the LDLR^{-/-} mouse overproduction of PCSK9 would not result in increased secretion of LDL,

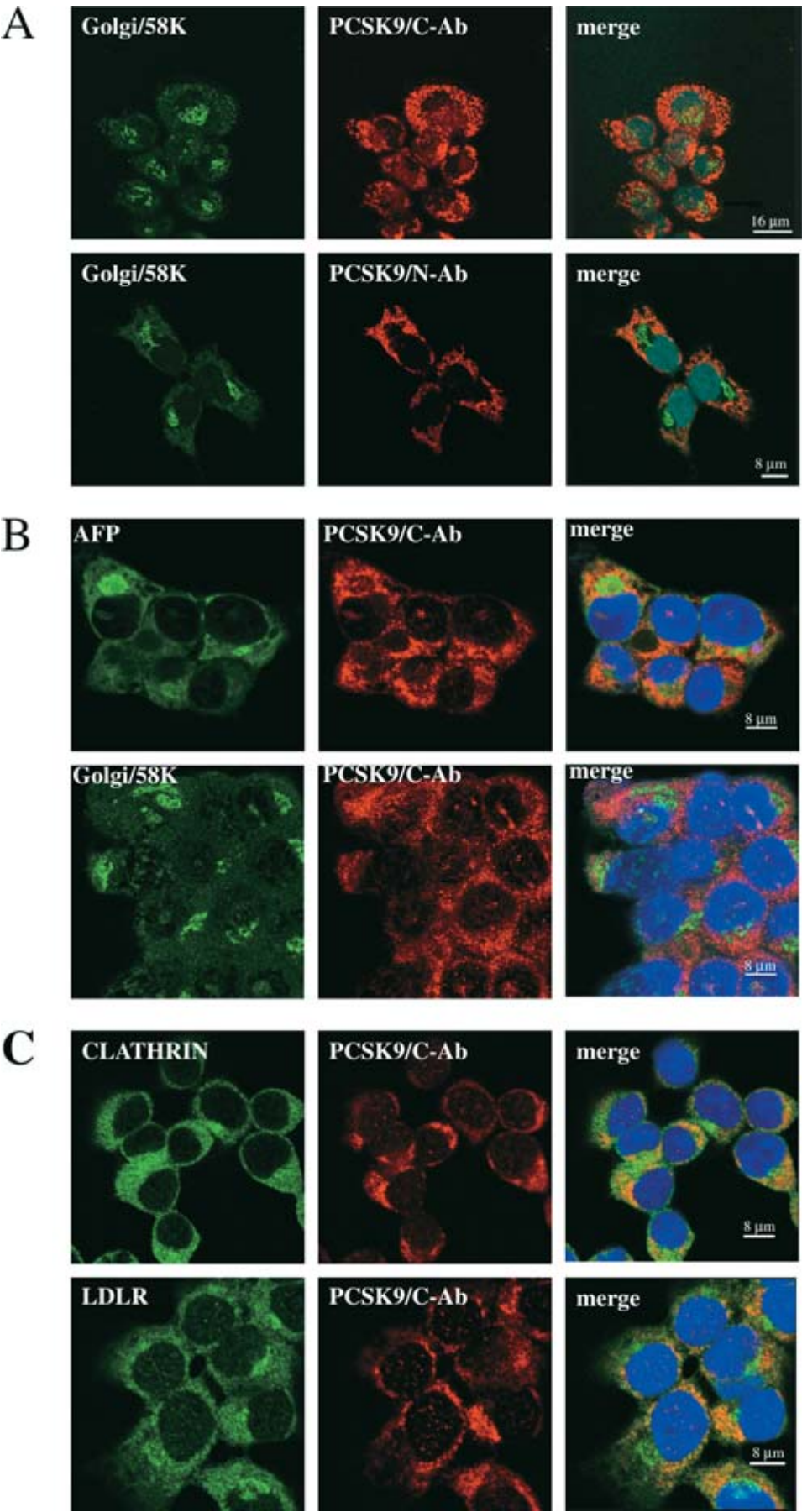


Fig. 5. Confocal immunofluorescence localization of PCSK9 in FAO-1 cells and rat hepatoblasts. (A) FAO-1 cells were grown on cover slips, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab or N-Ab) and anti-Golgi/58K, followed by anti-rabbit Cy3- (red) and anti-mouse Cy2 (green). Nuclei were contrastained with DAPI. Bars: 16 and 8 μ m, respectively. (B) Hepatoblasts were isolated as described in Materials and methods, grown on cover slips, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab) and anti- α -fetoprotein or anti-Golgi/58K, followed by anti-rabbit Cy3 (red) and anti-goat FITS (green) or anti-rabbit Cy3 (red) and anti-mouse Cy2 (green), respectively. Nuclei were contrastained with DAPI. Bar = 8 μ m. (C) FAO-1 cells were acetone fixed and incubated with antibodies against PCSK9 (C-Ab) and anti-clathrin heavy chain or anti-LDLR antibody followed by anti-rabbit Cy3 (red) and anti-mouse Cy2 (green) or anti-chicken FITS (green), respectively. Nuclei were contrastained with DAPI. Bar = 8 μ m.

Fig. 6. (A) Subcellular localization of PCSK9 in FAO-1 cells. Subcellular fractions of FAO-1 cells were obtained by ultracentrifugation as described in Materials and methods. The membranes with blotted protein were probed with different antibodies as shown. PNS, post nuclear supernatant; Pro-PCSK9 (pro-PCSK9), mature forms of the PCSK9 (PCSK9). Longer exposure of the heavy fractions, which reveals the proconvertase, is also given. Markers: Golgi, 58K and GM130; early endosomes, Rab 4; late endosomes, Rab 7; ERGIC, Ub-COPII (ubiquitinated COPII) molecules; clathrin-coated vesicles. The figure is representative of 3 independent fractionations. (B) Cell surface localization of PCSK9. Cell surface proteins were biotinylated as described in Materials and methods. Western blots were carried out with antibodies against PCSK9 (C-Ab) and anti LDLR. The membrane was consequently probed with the 2 antibodies. *, denotes unspecific band recognized by anti-PCSK9 (C-Ab) antibody (in both A and B).

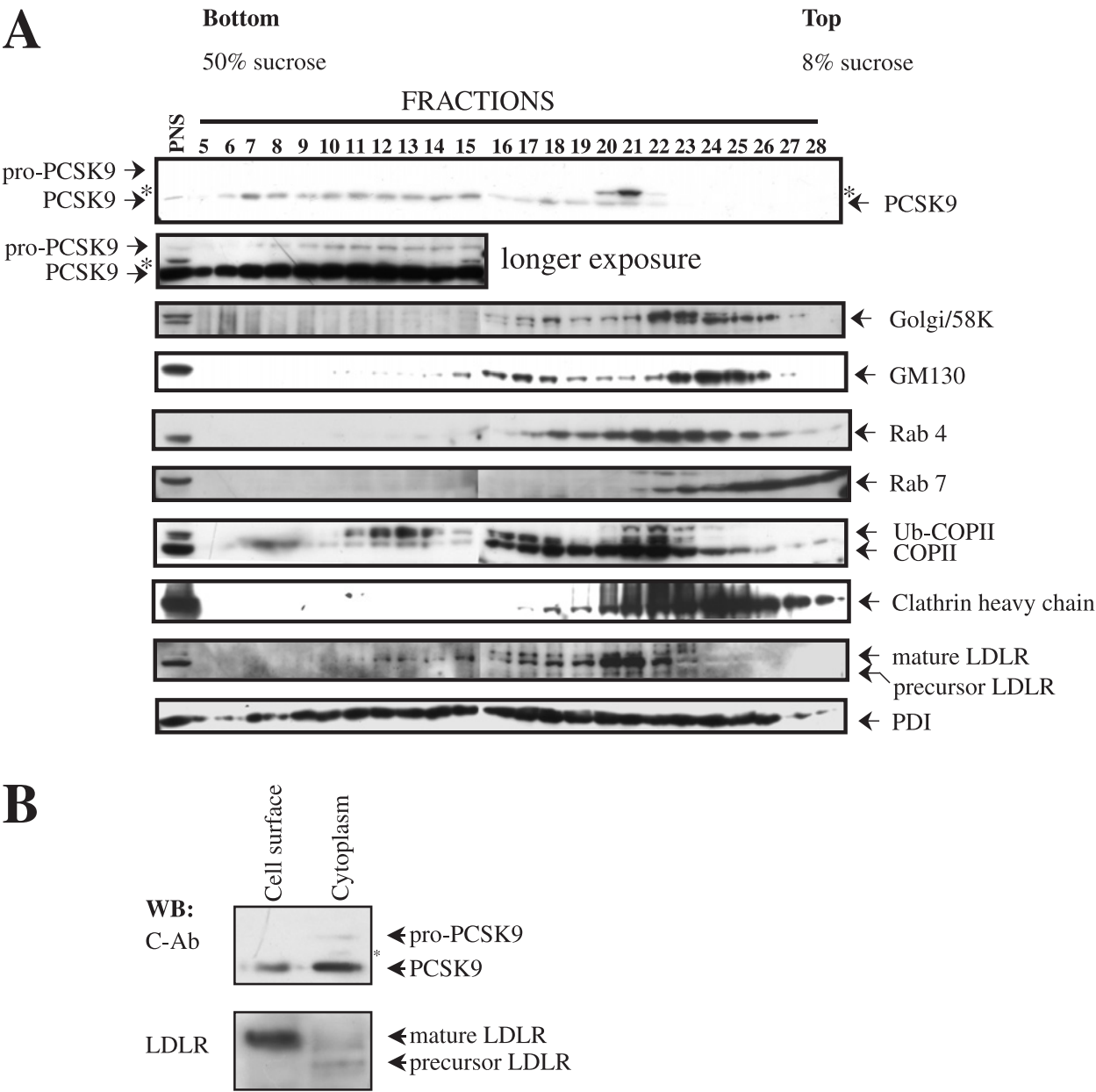
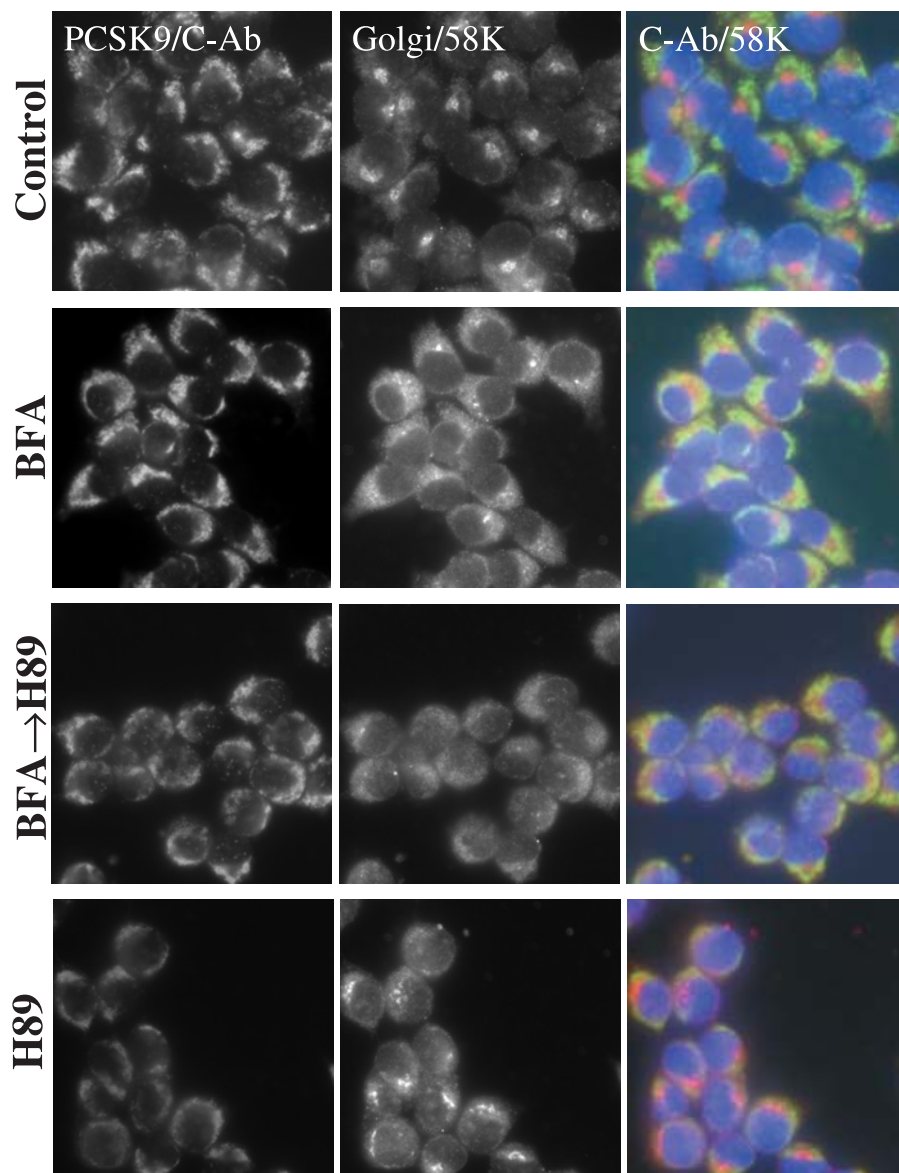


Fig. 7. Expression of PCSK9 after brefeldin A and H89 treatment. FAO-1 cells were grown on cover slips, treated with brefeldin A (BFA) for 40 min followed by H89 for 10 min (BFA → H89) or with H89 for 30 min (H89) as described in Materials and methods, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab) followed by anti-rabbit Cy2-conjugated (green) and monoclonal anti-Golgi/58K followed by anti-mouse Cy3-conjugated (red). The nuclei were contrasted with DAPI. Original magnification: $\times 600$.



which is exactly what was observed in these studies (Maxwell and Breslow 2004; Park et al. 2004).

Our work showed further that the suggested N-linked glycosylation of PCSK9 (15) occurs in the ER, as we did not detect the protein in the Golgi. Confocal immunofluorescent microscopy combined with ultracentrifugal subcellular fractionation and Western blot analysis revealed that the bulk of PCSK9 is localized in the ER: (i) the pro-enzyme shows diffuse distribution and it sediments with the ER membranes and (ii) most of the mature form also sediments with the ER. The remainder of the convertase is localized in transport vesicles in the ERGIC of the cell: (i) PCSK9 also appears in the lighter fractions of the gradient where COPII transport vesicles cosediment and (ii) when the cells are treated with brefeldin A and H89 the immunofluorescent signal for the convertase retreats into the ER. Some of PCSK9 is present

on the cell surface and is secreted: (i) experiments with biotinylation of the cell surface proteins demonstrate that small amounts of the convertase (about 10% of the total) reside on the cell surface and (ii) secreted convertase from FAO-1 cells was immunoprecipitated from the medium and from rat plasma–serum. We also found that some PCSK9 is in clathrin-coated vesicles, and this opens the possibility that some of the secreted PCSK9 is taken back into the cells by endocytosis.

In conclusion, our data show that PCSK9 is predominantly expressed in cells producing APOB, and its expression is regulated by cholesterol. PCSK9 is localized in the ER, where it is processed, and in the ERGIC of the cell but not in the Golgi cisternae. The convertase is secreted in the plasma. Our results also suggest that PCSK9 may upregulate APOB production in these compartments. Further functional

analyses of PCSK9 are of great importance for clarification of how this enzyme regulates LDL stability and APOB production and hence the plasma cholesterol level in fetal and adult liver.

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