ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and associated with phagocytosis

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Abstract ABCA7 is highly homologous to ABCA1 and mediates cellular cholesterol and phospholipid release by apolipoproteins when transfected in vitro. However, expression of ABCA7 was downregulated by increased cellular cholesterol while ABCA1 was upregulated, and the results were consistent by forced expression or downregulation of sterol-responsive/regulatory element (SRE) binding proteins (SREBPs). We analyzed the promoter of the ABCA7 gene and identified the new exon encoding 96 bp (mouse) and 95 bp (human) of the 5′ untranslated region and the transcription start site at 1,122 bp (mouse) and 1,260 bp (human) upstream of the initiation methionine codon. At 5′ upstream of this exon is the ABCA7 proximal promoter containing multiple binding sites of transcription factors for hematopoiesis and SRE of 9 bp at 212 bp (mouse) and 179 bp (human) upstream of the new exon. The apolipoprotein A-I-mediated lipid release was not influenced by suppression of the endogenous ABCA7 with small interfering RNA in mouse fibroblasts or by its increase in ABCA1-deficient mouse cells. In contrast, phagocytic activity was altered in parallel to the ABCA7 expression in these cells. When phagocytosis was induced, the messages increased for SREBP2, ABCA7, and other SREBP2-regulated proteins. The ABCA1 message decreased in this condition. We conclude that the ABCA7 gene is regulated by sterol in the opposite direction to ABCA1 through SRE/SREBP2 and that expression of ABCA7 by this regulation is associated with phagocytic activity.—Iwamoto, N., S. Abe-Dohmae, R. Sato, and S. Yokoyama. **ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and associated with phagocytosis.** *J. Lipid Res.* 2006. **47:** 1915–1927.

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Cholesterol, a unique and important lipid molecule that regulates many essential functions of the cell membrane, is not catabolized in somatic cells and therefore transported to the liver for conversion to bile acids. HDL plays a central role in this pathway. One of the major reactions for cellular cholesterol release is the biogenesis of HDL by removing cellular lipid with helical apolipoproteins (1, 2). This is also the main source of plasma HDL, as this reaction is defective in Tangier disease, familial HDL deficiency (3, 4). Mutations in the ABCA1 gene were identified in this disease (5–7), and this protein was shown to mediate the generation of HDL. ABCA7 has ∼50% homology to ABCA1 in amino acid sequence. When transfected, it supports the release of cellular phospholipid and cholesterol by helical apolipoprotein similar to ABCA1 in vitro (8–11), except that release of cholesterol is much less than that of phospholipids to generate cholesterol-poor HDL (8–13).

Consistent with its function, the transcription of the ABCA1 gene is positively regulated by cellular cholesterol through the liver X receptor (LXR) system (14). Oxysterol is an endogenous ligand for LXR and binds the DR4 element of the ABCA1 promoter (15, 16). ABCA7 mRNA and protein were both reportedly induced by modified LDL and downregulated by HDL_3 in macrophages differentiated from human monocytes (17), implicating its similar role to ABCA1 in cholesterol homeostasis. However, lipid release from macrophages remained normal in ABCA7-deficient mice, whereas adipose tissue mass and serum HDL cholesterol decreased somewhat only in females (18).

In contrast, the system of sterol-responsive/regulatory element (SRE) and sterol-responsive/regulatory element

binding protein (SREBP2) regulates many genes for sterol homeostasis by sensing the cellular cholesterol level, mostly in a negative feedback manner, including HMG-CoA reductase and the LDL receptor (19). SREBP2, however, reportedly downregulated ABCA1 transcription sensing of cellular sterol depletion, not by binding SRE but by binding the E-box element (20).

In this study, we demonstrate that endogenous ABCA7 regulates phagocytosis rather than HDL biogenesis by apolipoprotein, being regulated by the SREBP2 system. Transcriptional regulation of ABCA7 gene expression is negatively regulated by cell cholesterol, mediated by SREBP2 that binds to SRE in the promoter, in the opposite direction to that of ABCA1. We identified the new exon encoding the 5′ untranslated region (UTR) at ∼1,000 bp upstream of the ABCA7 exon 1, and the proximal promoter containing SREs located at a different region from what was proposed previously (21, 22).

MATERIALS AND METHODS

Reagents and antibodies

Mevalonic acid lactone, 25-, 22(R)-, and 22(S)-hydroxycholesterol, TO-901317, and (2-hydroxypropyl)-β-cyclodextrin were from Sigma. Sodium mevalonate was prepared in 10 mM potassium phosphate. Pravastatin, an inhibitor of HMG-CoA reductase, was provided by Sankyo Co., Ltd. Lipoprotein-deficient serum (LPDS) was prepared from fetal calf serum (FCS; Invitrogen) as a fraction of d > 1.23 g/ml. LDL was isolated from fresh human plasma. Antibodies against SREBP1 (SC-366) and SREBP2 (SC-8151) and nonimmune control IgG (SC-2027) were purchased from Santa Cruz Biotechnology. Monoclonal antibodies to mouse (MABI97-19) and human (MABI96-27) ABCA7 were generated in rats against peptides CPGRQHPKR-VSRFLEDPSSVETMI (mouse) and CPGLQHPKRVSQFLDDPST-AETVL (human), which correspond to the C terminus of each protein, respectively, at the MAB Institute (Yokohama, Japan). Rabbit antiserum against ABCA1 was produced as described previously (23, 24).

Cell culture and transfections

Fibroblasts BALB/3T3 (mouse) (25) and WI-38 (human) (26) and human cervical carcinoma cell line HeLa cells (27) were obtained from the RIKEN cell bank. The cells were cultured in DF medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) with 10% (v/v) FCS under 5% CO_2, 95% air at 37°C. Cells were subcultured in a 24-well tray and incubated for 24 h. The reporter luciferase plasmids (1 μg) and the phRL-TK (Promega) encoding Renilla luciferase (for normalization; 30 ng) were transfected using Lipofectamine 2000 (Invitrogen). After 6 h, the cells were washed with Dulbecco's phosphate-buffered saline and cultured in 10% LPDS/DF medium containing either 50 μM sodium mevalonate plus 50 μM pravastatin for a sterol(−) condition or 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol for a sterol(+) condition. The expression vectors of pSREBP-1a(1-487) and pSREBP-2(1-481), both constitutively active, were generated as described elsewhere (28). In cotransfection experiments, 100 pg of expression plasmid of SREBP-1a or SREBP-2 or the empty vector (mock) was supplemented. After incubation for 48 h, the cells were lysed and the luciferase activity was measured using the Dual-Luciferase Reporter System (Promega).

Mouse primary lung fibroblast

ABCA1-deficient mice were bred from ABCA1 heterozygote mice (DBA/1-Abca1tm1jdm/J) purchased from Jackson's Animal Laboratories (Stony Brook, NY), and DBA/1 wild-type mice were purchased from the local experimental animal supplier. The primary fibroblasts were prepared from fetal mouse lung. The experimental procedure was approved by the Animal Welfare Committee of Nagoya City University Graduate School of Medical Sciences according to institutional guidelines.

Cellular lipid release assay

Apolipoprotein A-I (apoA-I) was isolated from human plasma HDL as described previously (29). Cells were subcultured in six-well trays at a density of 3.0×10^5 for BALB/3T3 cells and at 80% confluence for primary lung fibroblasts in 10% FCS-DF medium. After a 48 h incubation, the cells were washed twice and incubated in 1 ml/well of the DF medium in the presence of 10 μg/ml apoA-I containing 0.1% BSA. Lipid content in the medium was determined by colorimetic enzymatic assay after 16 h (30). Cellular protein was dissolved in 0.1 N NaOH and determined with the BCA Protein Assay Kit (Pierce).

Immunofluorescence microscopy

BALB/3T3 cells were grown on Lab-Tek II chamber slides (Nalge Nunc International) at 50% confluence. For immunostaining, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed three times with 10 mM glycine, and permeabilized with 0.2% Triton X-100 for 5 min. After washing three times, the cells were blocked with 3% BSA for 1 h at 37°C, incubated with each of the primary monoclonal antibodies for 2 h at 37°C, and stained with 1 mg/ml Hoechst 33258 and FITC-conjugated anti-rat IgG secondary antibody after washing. The coverslips were mounted with a drop of FluorSave Reagent (Calbiochem). Fluorescent images were viewed with a LSM5 PASCAL laser scanning confocal microscope (Carl Zeiss).

Phagocytic assay

The cells were mixed with Fluoresbrite carboxylate yellow green microspheres (1.0 μm; Polysciences), and the culture plates were centrifuged for 3 min at 900 g. The cells were further incubated at 37°C for specified periods of time and thoroughly washed three times with the phosphate buffered saline to remove remaining extracellular beads. Phagocytic activity was measured with a FL600 fluorescent plate reader (Bio-Tek, Inc.) as the fluorescence intensity of the engulfed beads in the cells by subtracting the background of cells without phagocytosis.

Rapid amplification of 5′ cDNA ends

Total RNA was prepared from BALB/3T3 and WI-38 cells and used as templates. The reaction was carried out with the rapid amplification of 5′ cDNA ends (5′RACE) system version 2 (Invitrogen). For the first-strand cDNA synthesis, two separate primers located at exon 3 were used (mouse, 5′-CTTGTTTGGAAAGTG-3′ and 5′-ACCCCAGGCTTC-3′; human, 5′-CTTGTTTGGG-AAGTG-3′ and 5′-GAAAGCAGGTGTTG-3′). The same results were obtained from these primers. The 5′RACE fragments were generated with an anchor primer and exon 2-specific primers. The amplified products were cloned and sequenced using a PRISM 3100 (Applied Biosystems).

Luciferase reporter genes

The ABCA7 promoter region spanning −1,006, −415, −269, −196 to +84, +614, and +1,116 was amplified by ABCA7-specific primers using DBA/1 mouse spleen DNA as a template. The amplified fragments were inserted between the *Kpn*I and *Nhe*I restriction sites of pGL3 Basic vector (Promega), and the sequence was verified. The human ABCA7 promoter constructs were prepared similarly from human leukocytes. The reporter genes with mutated SREs were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The human ABCA1 promoter construct was a generous gift from Dr. H. Bryan Brewer, Jr. (National Heart, Lung, and Blood Institute, Bethesda, MD) (31).

RT-PCR

Total RNA was isolated and reverse-transcribed by SuperScript III (Invitrogen) with random oligonucleotide primers. Quantitative expression analysis was performed in an ABI PRISM 7700 (Applied Biosystems) using SYBR Green technology. The following PCR primers were used for amplification of mouse and human RNAs: ABCA7, 5′-GCCAGTATGGAATCCCTGAA-3′ (forward) and 5′-ATGGAGACACCAGGAACCAG-3′ (reverse); ABCA1, 5′-TCCCGGCGAGGCTCCCGGTGT-3′ (forward) and 5′-CAGCTCTTGGGCCAGGCCCC-3′ (reverse); SREBP1, 5′-AA-CCAGAAGCTCAAGCAGGA-3′ (forward) and 5′-TCATGCCCTC-CATAGACACA-3′ (reverse); SREBP2, 5′-GTGGAGCAGTCTCA-ACGTCA-3′ (forward) and 5′-TGGTAGGTCTCACCCAGGAG-3′ (reverse); HMG-CoA reductase, 5′-GATCGAAGGACGAGGA-AAGAC-3′ (forward) and 5′-CACATCACCAGTTTCCAGCTT-3′ (reverse); LDL receptor, 5′-GGGTGTGCGTGGCAGCCCCG-3′ (forward) and 5′-CAGGTGGCCACCGCGCAGTGC-3′ (reverse); GAPDH, 5′-CCATGGAGAAGGCCGGGGCCC-3′ (forward) and 5′-GGGCAGCCCCACGGCCATCAC-3′ (reverse); 18S rRNA, 5′-GCAATTATTCCCCATGAACA-3′ (forward) and 5′-GGCCTC-ACTAAACCATCCAA-3′ (reverse). Results were normalized to GAPDH or 18S rRNA.

Western blot analysis

Membrane and nuclear proteins were prepared as described previously (23). Western blot analysis was carried out with specific antibodies.

RNA interference

Small interfering RNA (siRNA) duplexes specific for SREBP1 (5′-AAGAAACGUGUCAAGAAGUGCAGGG-3′), SREBP2 (5′-AA-UGAUCUGAGGUUGCACCAGGACC-3′), or control (5′-UAGU-GAAGACAGUCACUCGGGAAGC-3′) were obtained from Invitrogen and transfected into BALB/3T3 cells using Lipofect-amine 2000 for 48 h before further analysis. Those for ABCA7 were 1 (5′-UUCGAAUCUUGAAUCGUAGGUGGCC-3′) and 2 (5′-UAAGCACUAAUACCAGCAACGCAGC-3′).

Gel shift assay

The SREBP2 expression plasmid was transfected to BALB/3T3 cells for 48 h, the cells were lysed, and nuclear extracts were prepared. A double-stranded DNA fragment corresponding to the −197 to −239 sequence of the mouse ABCA7 promoter was 3′ end-labeled with a digoxigenin-11-ddUTP according to the DIG Gel Shift Kit, second generation (Roche Applied Science). In competition assays, 100-fold excess of unlabeled 42 bp DNA fragments containing the SRE or the mutated SRE was added to

the labeled probe. The bands were detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assays were performed as described previously (32). Cells were cultured for 48 h. After cross-linking of proteins with 1% formaldehyde, the nuclear extract was prepared and sonicated. For immunoprecipitation, 2 μg of SREBP1/2-specific antibody or nonimmune IgG was incubated overnight at 4°C with the nuclear proteins, and the DNA to which the antibody binds was purified with protein A-Sepharose 4B beads (Santa Cruz Biotechnology). The mouse ABCA7 promoter containing SRE was amplified with the specific
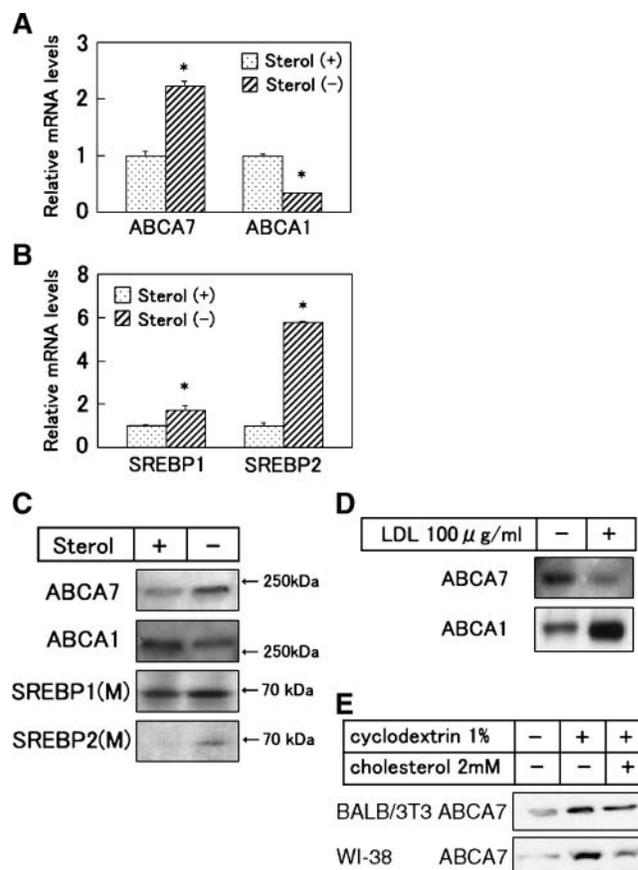

Fig. 1. ABCA7 mRNA and protein in cellular cholesterol depletion. A, B: Expression of the mRNA analyzed by quantitative RT-PCR. BALB/3T3 cells were cultured in medium containing 10% lipoprotein-deficient serum (LPDS) with either 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol [sterol(+)] or 50 μM sodium mevalonate plus 50 μM pravastatin [sterol(−)] for 48 h. The mRNA level of each gene was determined. GAPDH and 18S rRNA were used as standards. C–E: Expression of the ABCA7 protein. C: Cells were grown as described above. The membrane, 100 μg of protein (for ABCA1 and ABCA7), and the nuclear fraction, 40 μg [for sterol-responsive/regulatory element binding protein 1/2 (SREBP1/2)], were analyzed by Western blot. D: Primary lung fibroblasts of mice were treated with or without (unmodified) 100 μg/ml LDL for 24 h. ABCA7 and ABCA1 in the membrane were analyzed by Western blot. E: BALB/3T3 cells and WI-38 cells were cultured with 1% (2-hydroxypropyl)-β-cyclodextrin for 4 h (lane 2) or cyclodextrin plus 2 mM cholesterol (lane 3), and ABCA7 was analyzed by Western blot. Data represent means ± SD for three samples. * $P < 0.001$.

primers 5′-AGTCCTTGGGGAGAGGAGAC-3′ (forward) and 5′-GTTTGGCTTCACTGGGACAC-3′ (reverse).

RESULTS

Regulation of ABCA7 expression by sterol

The regulation of ABCA7 by sterol was examined. BALB/3T3 cells were cultured in the sterol(+) or sterol(−) condition. In the sterol(−) condition, the ABCA7 mRNA and protein both increased from the sterol(+) condition, whereas those of ABCA1 decreased (**Fig. 1A, C**). Response to sterol of the mRNA and protein levels of SREBP1 and SREBP2 was parallel to that of ABCA7 (Fig. 1B, C). The increase of SREBP1 by sterol depletion may indicate predominant expression of SERBP1a (95.3%) over SERBP1c (4.7%) in this cell line. ABCA7 markedly decreased when loading LDL (Fig. 1D), whereas ABCA1 increased. ABCA7 markedly increased when cellular cholesterol was depleted by 1% (2-hydroxypropyl)-β-cyclodextrin, and this upregulation was reversed by replenishing cellular sterol (Fig. 1E).

When BALB/3T3 cells were transfected with SREBP1a or SREBP2, ABCA7 mRNA was upregulated by 19% (SREBP1a) and 50% (SREBP2), similar to the messages of the LDL receptor and HMG-CoA reductase (**Fig. 2A**), consistent with other findings. ABCA7 protein was also increased by the expression of SREBPs and the upregulation of their mature forms (Fig. 2B). In contrast, the expression of ABCA1 mRNA was inhibited by SREBP2. To confirm that ABCA7 transcription is a target of SREBPs, the expression of endogenous SREBP1, SREBP2, and SREBP1/2 was inhibited by siRNA by 80.1, 76.4, and 66.6/73.3%, respectively (data not shown). ABCA7 mRNA increased by 70.6% in the sterol(−) condition in control cells

(Fig. 2C), and this increment was reduced by knockdown of SREBP1 (21.1%), SREBP2 (18.0%), and SREBP1/2 (7.8%). ABCA7 mRNA was also diminished by the suppression of SREBPs in the sterol(+) condition (Fig. 2C). ABCA1 mRNA was markedly increased in BALB/3T3 cells by a synthetic LXR agonist, TO-901317, and an endogenous LXR agonist, 22(R)-hydroxycholesterol, but neither influenced ABCA7 mRNA expression (Fig. 2D). These results indicate that ABCA7 transcription is regulated by SREBPs but not by LXR.

Analysis of the promoter of the ABCA7 gene

To identify the 5′ end of the ABCA7 mRNA and the ABCA7 proximal promoter region, 5′RACE was performed for total RNA purified from BALB/3T3 and WI-38 cells as templates. The mouse and human ABCA7 genes reportedly contain 45 and 46 exons, respectively, and each exon 1 encodes the initiation methionine codon (21, 22). However, the new exon, exon X, was found encoding 96 bp (mouse) and 95 bp (human) of 5′ UTR at 1,019 bp (mouse) and 1,028 bp (human) upstream of exon 1. The organization sizes of exon X and intron X (between exons X and 1) are similar between the two species, but the length of the 5′-UTR at the mouse exon 1 is shorter than that of human (7 vs. 137 bp) (**Fig. 3A, B**). The function of the 5′-flanking region of exon X is unknown, whereas previous reports indicated that intron X is the putative promoter (7, 25). The 320 bp element of the end of intron X was highly conserved between mouse and human (62.0% vs. 44.4%) (Fig. 3C). To determine the proximal promoter, we prepared constructs containing various regions (Fig. 3D) and used the 2,121 bp construct containing the 1,006 bp 5′-flanking sequence, the 96 bp exon X, and the 1,019 bp intron X (construct 5) as a standard . The
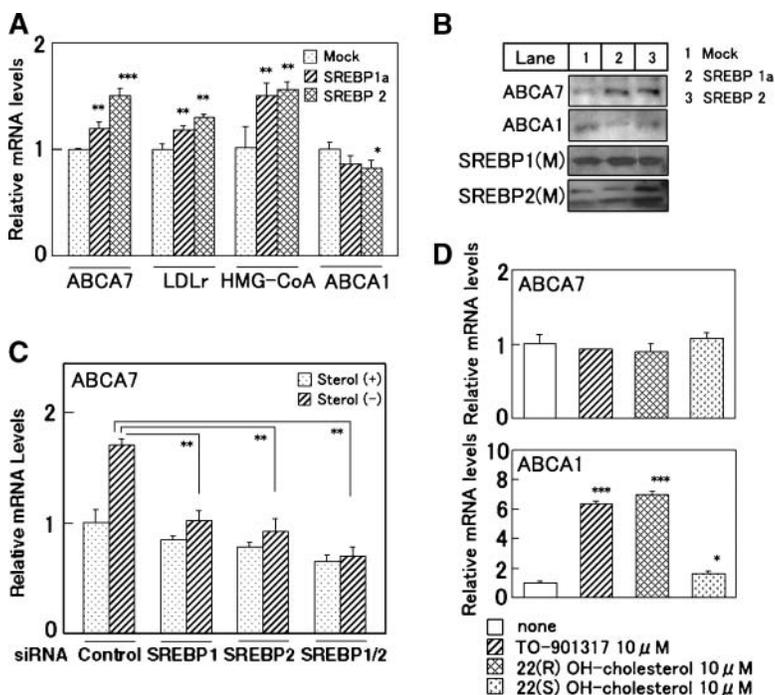
Fig. 2. Transcription of the ABCA7 gene and SREBPs. A: Upregulation of the ABCA7 gene by SREBPs. BALB/3T3 cells on six-well plates were transfected with 1 μg of the expression vector of SREBP1a and SREBP2 for 48 h, and total RNA was purified. The mRNA transcriptional level was determined by quantitative RT-PCR for ABCA7, the LDL receptor (LDLr), HMG-CoA reductase (HMG-CoA), and ABCA1. B: BALB/3T3 cells were treated as described above, and proteins were analyzed by Western blot. C: BALB/3T3 cells on six-well plates were transfected with 250 pmol of small interfering RNA (siRNA) of each SREBP for 6 h. The cellular sterol condition was altered as described for Fig. 1A. ABCA7 mRNA expression was analyzed by quantitative RT-PCR. D: Expression of the mRNAs of ABCA1 and ABCA7 was analyzed in BALB/3T3 cells treated with the compound indicated for 16 h. Results represent means ± SD. ***P < 0.001, **P < 0.01, *P < 0.05.

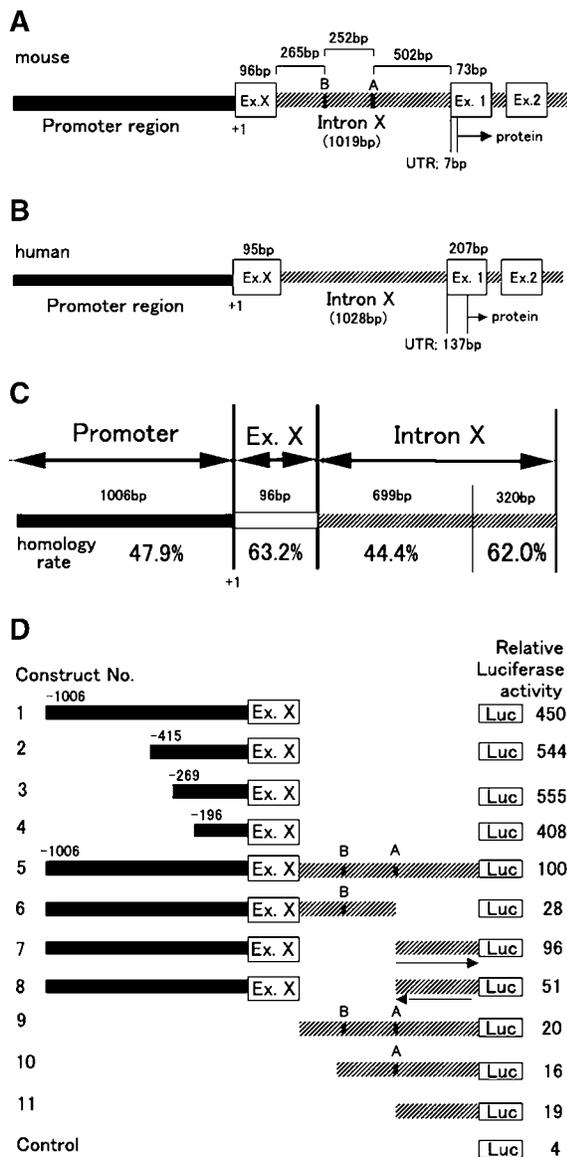**Fig. 3.** Analysis of the ABCA7 promoter. A, B: Exon X is the newly identified segment by rapid amplification of 5′ cDNA ends (5′RACE). The genomic organization of the 5′-flanking region of exon 1 in the mouse (A) and human (B) ABCA7 genes is shown. The predicted transcription start site at exon X is marked as position +1. A, *Apa*I; B, *Bgl*II; Ex., exon; UTR, untranslated region. C: The homologous sequence rate at each region in the mouse and human ABCA7 genes is indicated as a percentage. D: Schematic diagram of the various DNA constructs for the reporter assay (1–11) containing exon X, intron X, the promoter region, and control pGL3 basic vector. Relative expression levels estimated as luciferase activity are given in the right column. The arrows in the intron X region show polarity.

luciferase activity of the 5′-flanking region of exon X was 22.5 times higher than that of intron X (construct 9 vs. 1), but the previously predicted promoter region showed very low activity (constructs 10 and 11 vs. 1). It was thus concluded that the 5′-flanking region of exon X is the proximal promoter. We further examined how intron X affects the transcription of the ABCA7 gene. By deleting the

502 bp element at the end of intron X (construct 6), luciferase activity was decreased by 4-fold. Insertion of this 502 bp element in the reverse orientation reduced reporter activity by half (construct 8) but did not change it in the correct polarity (construct 7). Therefore, the 502 bp element at the end of intron X is not a classical/conventional enhancer, but it may work as a positive modulator.

Figure 4 shows the newly identified ABCA7 promoter sequences of mouse and human. In both species, a typical TATA box is not detectable, but a GC box and a CAAT box are located between −20 and −70 bp of both promoters. Interestingly, the ABCA7 promoter contains multiple binding motifs for hematopoietic development and differentiation, such as AML1a, LYF1, MZF1, GATA, and Ikaros2, all of which are highly conserved between mouse and human.

Identification of the functional SRE in the promoter of the ABCA7 gene

Six candidate sites of SRE were identified in the newly proposed mouse promoter region between −1 and −1,006 bp (**Fig. 5A**). To find which is critical for SREBP binding, several deletion constructs were prepared for the mouse ABCA7 reporter gene. By deleting from −1,006 to
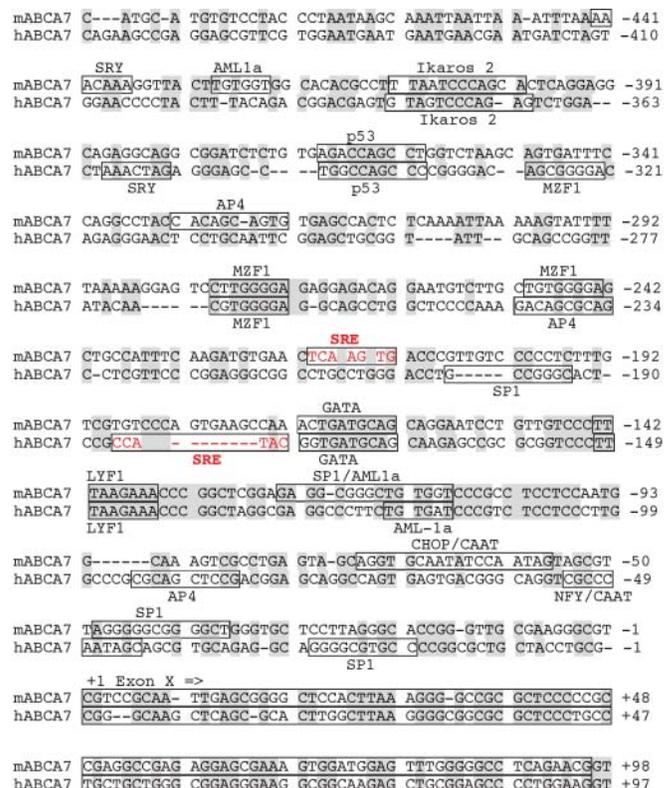

Fig. 4. Mouse (m) and human (h) ABCA7 promoter sequences. Homologous sequences are highlighted. The newly identified exon X is boxed. The potential binding sites for transcription factors are boxed and marked. Sterol-responsive/regulatory elements (SREs) are indicated in red. The transcription start site is predicted by TSSG, TSSW, and DBTTS (Data Base of Transcription Start Sites), which expected 5 bp upstream of the experimentally observed site.
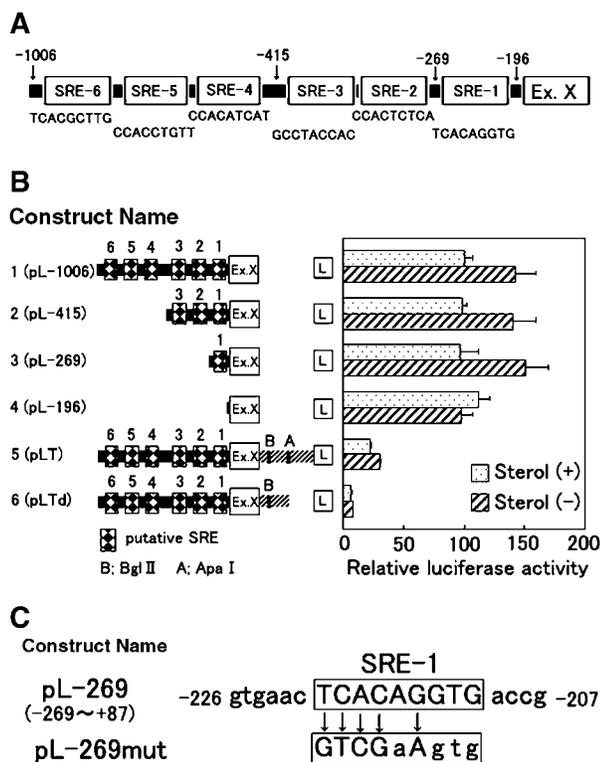
Fig. 5. Identification of the SRE that regulates ABCA7 transcription. A: Schematic diagram of the putative SRE motifs in the mouse ABCA7 promoter. B: Six reporter genes with different lengths of the promoter region were constructed. BALB/3T3 cells were transfected with either one of these reporter constructs for the ABCA7 promoter or the control vector. The cells were cultured for 48 h either in the sterol(+) or sterol(−) condition. Luciferase activity was measured and normalized for phRL-TK. The hatched boxes indicate the putative SRE and are numbered from the one nearest to the transcription start site. Data represent means ± SD. C: The mutant sequence in the mouse SRE site (−220 to −212, indicated as a box) is illustrated. The arrowed nucleotides are those mutated in the pL-269mut construct.

Fig. 6. Regulation of ABCA7 expression by sterol and SREBP. A: BALB/3T3 cells on 24-well plates were transfected with 1 μg of the indicated construct and incubated for 48 h either in the sterol(+) or sterol(−) condition. B: BALB/3T3 cells were cotransfected with 1 μg of the indicated reporter gene constructs and 100 pg of the expression plasmids of SREBPs for 6 h and cultured in the medium containing 10% LPDS for 48 h, and reporter gene activity was determined. C: BALB/3T3 cells on 24-well plates were cotransfected with 200 ng of the pL-269 reporter gene and 10 pmol of siRNA of each interference primer and incubated for 48 h either in the sterol(+) or sterol(−) condition. Relative luciferase activity was calculated for each control in the sterol(+) condition. Data represent means ± SD for three samples. ** $P < 0.01$.

−415 and −269 (constructs pL-1006, pL-415, and pL-269), the promoter activity was increased under the sterol-depleted conditions. This upregulation was also recognized with the constructs pLT and pLdT containing intron X. However, the pL-196 truncated construct with no SRE (construct pL-196) lost the enhancement in the sterol(−) condition (Fig. 5B). These results indicated that the region between −269 and −196 is essential for the transcriptional regulation by SREBPs and that the putative SRE-1 (TCACAGGTG) is the SREBPs binding site in mouse. To ensure this, luciferase activity was examined for the mutant fragment with GTCGAAGTG sequences (pL-269mut) instead of TCACAGGTG (−220 to −212 bp) (Fig. 5C).

With the truncated pL-196 and mutated pL-269mut constructs, the upregulation of the promoter disappeared (**Fig. 6A, B**). The pL-415 and pL-269 reporter gene activities were significantly increased by both sterol depletion and cotransfection of SREBP2 but not by SREBP1a (Fig. 6B). The effect of sterol depletion was also abolished when endogenous SREBPs were knocked down by siRNA (Fig. 6C).
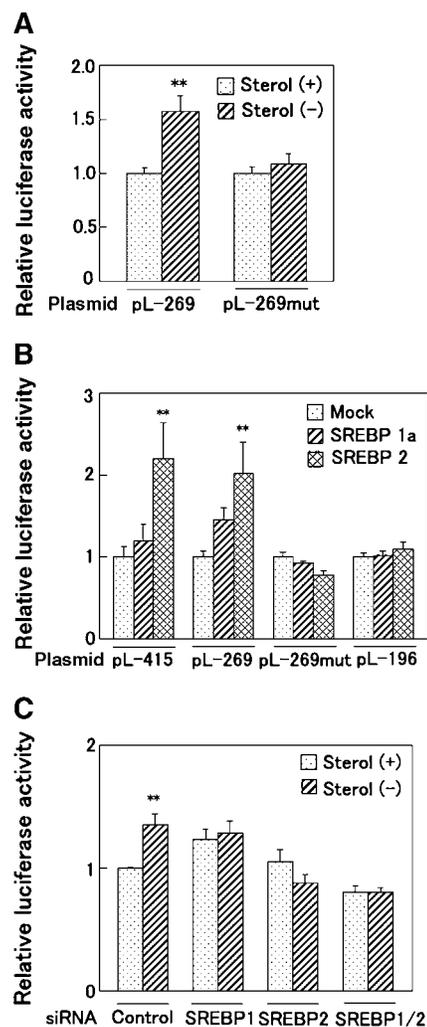
Gel shift assay confirmed SREBP2 binding to the putative SRE-1 in vitro (**Fig. 7A**). The DNA-protein complex band was observed in the presence of recombinant SREBP2 (lane 2). Competition with the unlabeled SRE-1 (lane 3) or its mutated probe (lane 4) showed that SRE-1 is a specific site for SREBP2 binding. In the supershift assay, the DNA-protein complex band disappeared, but the apparent supershift band was not detectable by SREBP2 antibody (data not shown). Finally, the ChIP assay showed that SREBP2 associates with the ABCA7 promoter more strongly in the sterol(−) condition than in the sterol(+) condition (Fig. 7B), suggesting that endogenous SREBP2
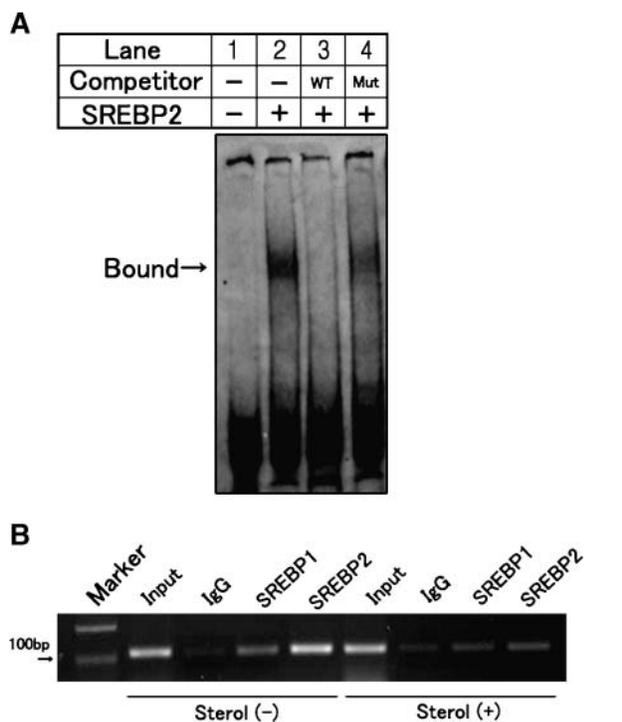
Fig. 7. Interaction of SREBP2 and the SRE in the ABCA7 promoter. A: Gel shift assay of the nuclear extract from active SREBP2-transfected BALB/3T3 cells. The oligonucleotide probes representing the −197 to −239 sequence of the ABCA7 promoter were labeled with digoxigenin. Mut, SRE mutant probe; WT, wild-type probe. B: BALB/3T3 cells were incubated for 48 h either in the sterol(+) or sterol(−) condition. Each sample was immuno-precipitated by specific antibodies. DNA was eluted and purified. PCR was performed using mouse ABCA7 promoter-specific primers (product size, 114 bp).

binds SRE-1 of the mouse ABCA7 promoter. These results indicate that SREBP2 binds the SRE site (−220 to −212 bp) in the ABCA7 promoter and that this association is essential for the transcriptional regulation of the ABCA7 gene by sterol.

In the human ABCA7 promoter, the putative SRE was located between −188 and −179. Three reporter constructs were prepared to examine whether this position is responsible for SREBP2 binding (**Fig. 8A**). In the sterol-depleted condition, the luciferase activity of the phL-293 construct increased by 50%, but the promoter activities of the phL-293mut (SRE mutated) and phL-153 (deleted) constructs were not changed by alteration of the sterol condition. Depletion of sterol decreased ABCA1 promoter activity (Fig. 8B). Overexpression of SREBP2 increased the activity of the promoter construct phL-293 but not phL-293mut or phL-153 (Fig. 8C). These results show that the SRE between −188 and −179 bp in the human ABCA7 promoter is the SREBP2 binding site.

To examine whether the newly identified exon is expressed in other cells and tissues, we performed RT-PCR using exon X-specific primers. Exon X was transcribed in J774 and RAW264 macrophage cells in addition to BALB/3T3 cells and also in the tissues expressing ABCA7 protein,
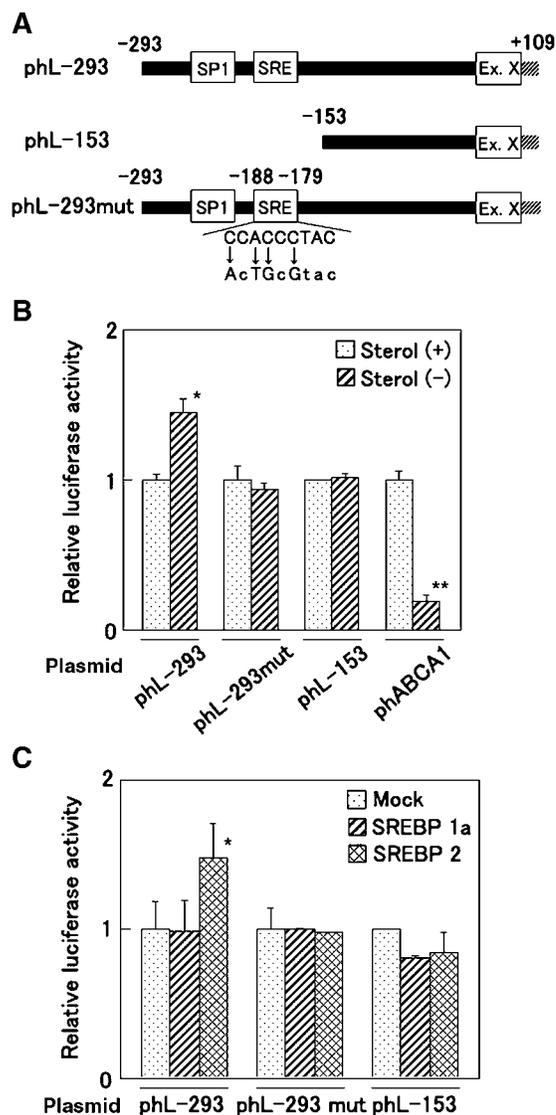

Fig. 8. SREBP2 binds to the SRE in the human ABCA7 promoter. A: Schematic diagram of the putative SRE motifs in the human ABCA7 promoter. The phL-293 (−293 to +109) and phL-153 (−153 to +109) reporter genes contain the human ABCA7 promoter sequences. In the phL-293mut construct, the putative SRE was mutated as described. B: HeLa cells were transfected with 1 μg of the indicated construct and incubated for 48 h in either the sterol(+) or sterol(−) condition. phABCA1 contains the E box and DR4 element in the human ABCA1 promoter. C: HeLa cells on 24-well plates were cotransfected with the reporter constructs (1 μg) and the expression plasmids of SREBPs (20 pg) for 6 h and cultured in medium containing 10% LPDS for 48 h. Data represent means ± SD. ** $P < 0.001$, * $P < 0.01$.

such as thymus and spleen (**Fig. 9A**). The human macrophage cell line THP-1 also contained the new noncoding exon (Fig. 9B).

ABCA7 and phagocytosis

We evaluated the function of endogenous ABCA7 at the cellular level. Endogenous ABCA7 was downregulated by 76.2% at the mRNA level by siRNA in BALB/3T3 cells (**Fig. 10A**), and cellular lipid release from apoA-I was
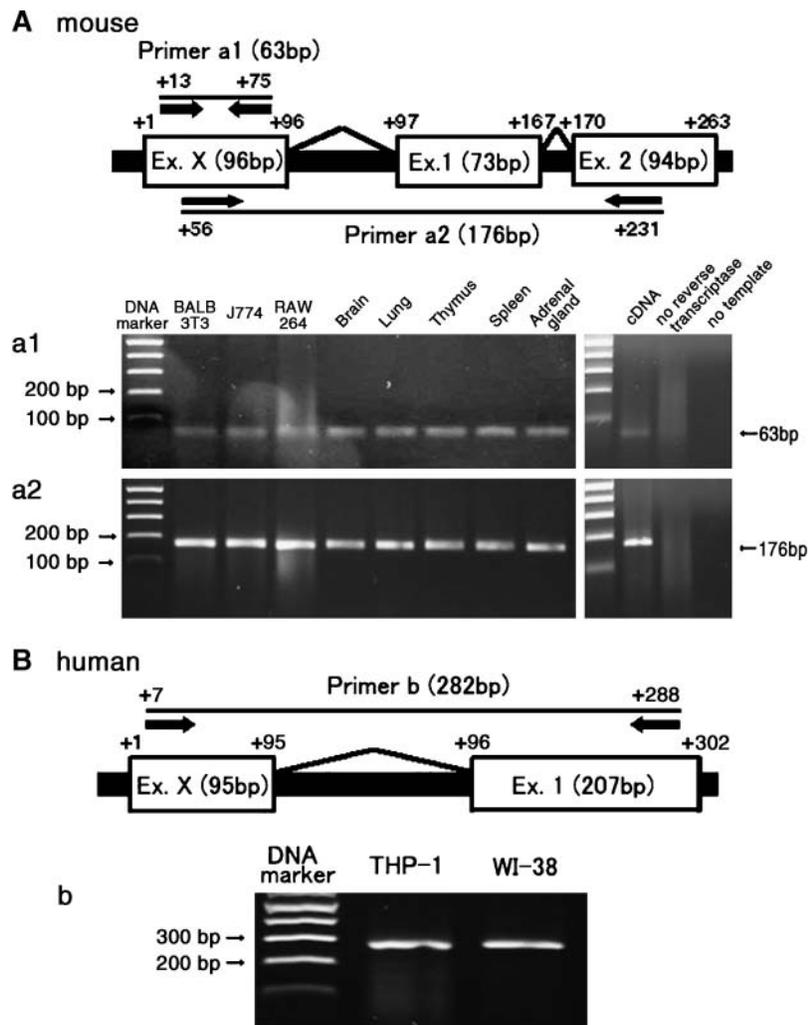
Fig. 9. The newly identified exon X was transcribed in several cells and tissues of mouse (A) and human (B). Total RNAs were purified and transcribed to prepare cDNA by RT-PCR. PCR was performed with each cDNA template by specific primers of a1, a2 (mouse), and b (human) with exon X sequences. Predicted PCR product sizes (a1, 63 bp; a2, 176 bp; b, 282 bp) are indicated in the schematic diagrams. Primers were as follows: a1 (forward, AGCGGGGCTCCACTTAAA; reverse, CATCCACTTTCGCTCCTCTC), a2 (forward, GAGAGGAGCGAAAGTGGATG; reverse, CGGACAGCCACTAGGATGA), b (forward, CAAGCTCAGCGCA-CTTGG; reverse, CGGCGATACATGAAATTCTTC). Position +1 indicates the transcription start site, and the numbers (mouse, +1 to +263; human, +1 to +302) indicate positions in cDNA from the transcription start site. Contamination of genomic DNA and the possibility that the lower molecular weight PCR product bands were primer-dimers were both ruled out by running control experiments for the RNA isolated from BALB/3T3 cells without reverse transcriptase or without templates (right panels of a1 and a2).

examined (Fig. 10B). Release of cholesterol or phospholipid was not influenced in this condition.

CED-7, a similar protein to ABCA1 in *Caenorhabditis elegans*, was shown to be required for phagocytosis (33), suggesting the involvement of ABCA proteins in the regulation of phagocytosis. When ABCA7 was downregulated by its siRNA in BALB/3T3 cells, their phagocytic activity to the latex beads decreased significantly (Fig. 10C). The phagocytic rate decreased by 50% by two different siRNAs at every incubation time examined (Fig. 10D). These results thus indicate that ABCA7 regulates type II phagocytic function that is not mediated by the Fc receptor.

Figure 11A shows that cellular cholesterol and phospholipid were higher during phagocytosis in control cells

but not in ABCA7 siRNA-transfected cells. Phagocytosis increased the SREBP1/2 mRNA at an early stage (2 h) and then decreased gradually (Fig. 11B). The mature SREBPs increased until 6 h (Fig. 11C). The mRNA of the SREBP-regulated genes, the LDL receptor and HMG-CoA reductase, increased up to 6 h (Fig. 11B). Although ABCA7 protein and its mRNA increased in phagocytosis (Fig. 11B,C), ABCA1 expression was inhibited by half (Fig. 11B). These results are all consistent with the view that ABCA7 transcription is enhanced in phagocytosis as a result of the activation of SREBPs. Finally, the reporter gene assay was conducted for the ABCA7 promoter in the presence and absence of phagocytosis (Fig. 11D). The activity of the mouse ABCA7 promoter constructs pL-1006, pL-415, and pL-269
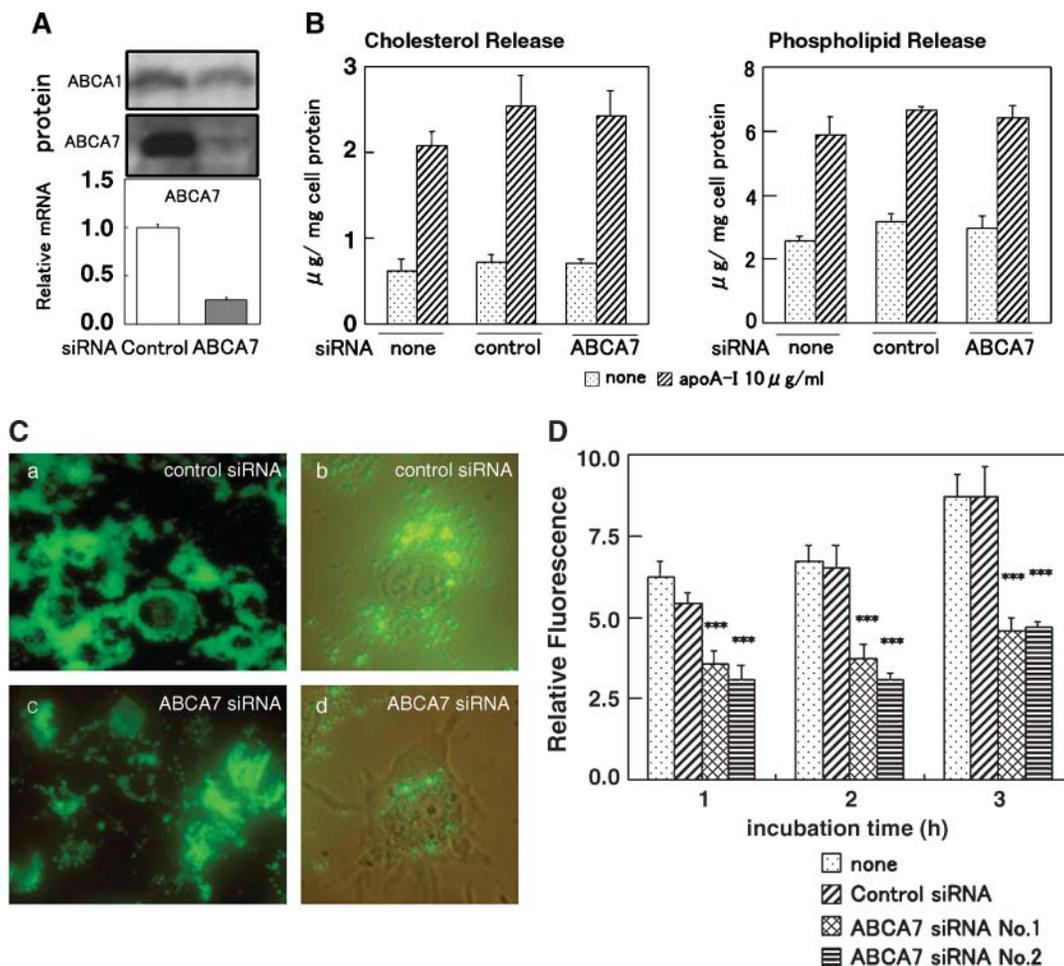
Fig. 10. Activation of ABCA7 and phagocytosis. A: Downregulation of ABCA7 by siRNA. BALB/3T3 cells were transfected with scramble control or mouse ABCA7-specific siRNA. After 48 h of incubation, ABCA1 and ABCA7 were analyzed by Western blotting, and ABCA7 mRNA was analyzed by quantitative RT-PCR. B: Apolipoprotein A-I (apoA-I)-mediated lipid release was examined with the cells treated with siRNA of ABCA7 described in the text. After 48 h of transfection, the medium was changed to serum-free DF medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) containing 0.1% BSA with or without 10 μg/ml apoA-I. The cells were incubated for 16 h, and lipid concentration in the medium was measured by enzymatic assay. C: Phagocytic activity of the cells. BALB/3T3 cells at 80% confluence on 96-well plates for 24 h were transfected with each siRNA primer. After 48 h of transfection, cultured medium was changed to 10% fetal calf serum (FCS)-DF medium with latex beads (50 μg/well), and the cells were imaged by Nikon Eclipse TE300. a, c: Low-magnification views (20×, green fluorescence) at 1 h of incubation. b, d: High-magnification views (60×, green fluorescence with phase contrast). D: Quantitative measurement of phagocytic activity when ABCA7 was downregulated by siRNAs 1 and 2 to 32.4 ± 1.0% and 31.9 ± 1.2%, respectively. Cells were mixed with latex beads (200 μg/well), and cellular fluorescence intensity was determined as described in Materials and Methods. Data represent means ± SD. ***$P < 0.001$ compared with control siRNA.

increased significantly in response to phagocytosis. However, pL-269m, in which SRE was mutated, and pL-196, which lacks SRE, did not respond to phagocytosis. These results were similar to the response of these reporter genes to sterol depletion (Figs. 5B, 6A). Thus, they indicate that ABCA7 in mammalian cells promotes phagocytosis similarly to CED-7 in *C. elegans* and that its gene expression is enhanced by phagocytosis.

We previously reported that ABCA7, when transfected and overexpressed in HEK293 cells, supported the apolipoprotein-mediated release of cellular lipid and the biogenesis of HDL (8–11), similar to ABCA1. However, endogenous ABCA7 was unlike to mediate this reaction (Fig. 10A, B). Accordingly, fibroblasts of the ABCA1-deficient mouse were examined because ABCA7 protein increased in this model animal (**Fig. 12A**). Figure 12B shows that fibroblasts of the ABCA1-deficient mouse do not release cholesterol or phospholipid by apoA-I in spite of the increase of ABCA7. In contrast, phagocytic activity increased significantly in the ABCA1-deficient fibroblasts (Fig. 12C), consistent with the findings in cells from Tangier disease patients (34). Thus, endogenous ABCA7 functions not to mediate HDL biogenesis but rather to regulate phagocytic function.

Figure 12D demonstrates different subcellular localization of endogenous ABCA1 and ABCA7 in BALB/3T3 cells by immunofluorescence microscopy. In contrast to ABCA1, which was localized predominantly in the plasma
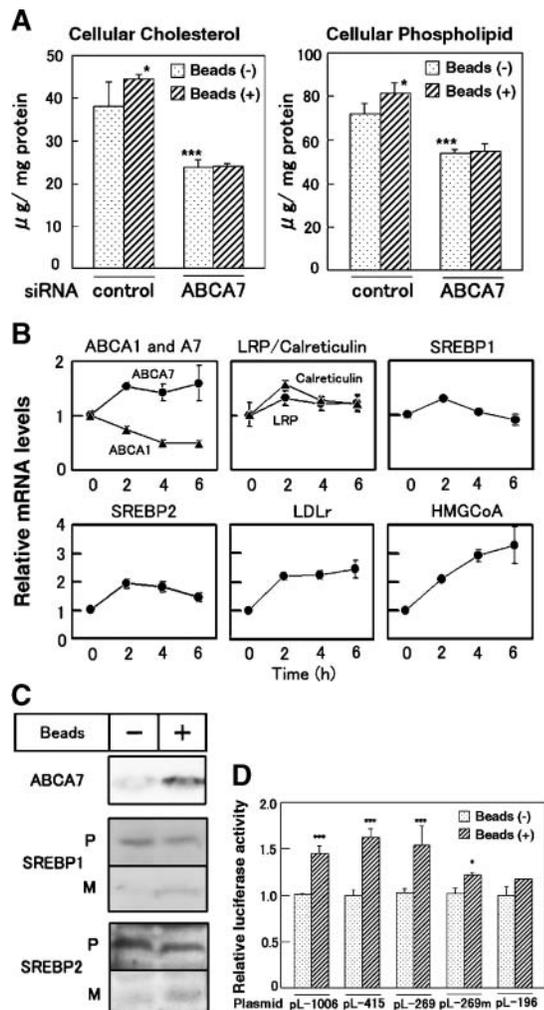
Fig. 11. ABCA7, phagocytosis, and SREBPs. A: Effect of phagocytosis on cellular lipid. The cells were incubated with or without latex beads (400 μg/well) for 3 h and washed. Cellular lipids were extracted by *n*-hexane-isopropanol (3:2) and measured by enzymatic assay. Results represent means ± SD for three samples. *** $P < 0.001$, * $P < 0.05$ from controls without beads. B: Effect of phagocytosis on the expression of SREBP-related genes. Cells were placed on six-well plates at 80% confluence. After 48 h, cultured medium was switched to 10% FCS-DF medium with latex beads (400 μg/well) and centrifuged for 3 min at 900 *g*. The cells were incubated for the indicated periods of time, and mRNA was analyzed by quantitative RT-PCR and normalized for GAPDH mRNA. Results represent means ± SD. C: Activation of SREBPs and increase of ABCA7 by phagocytosis. Cells were cultured for 6 h with and without latex beads (2.5 μg/dish) on 100 mm plates and harvested. The membrane fraction (100 μg of protein) and nuclear fraction (80 μg of protein) were analyzed by 6% and 8% SDS-PAGE, respectively. P and M denote the precursor and mature nuclear forms (120 and 70 kDa) of SREBPs, respectively. D: Activation of the ABCA7 promoter by phagocytosis. Cells were incubated on 24-well plates and transfected with 1 μg/well of the indicated mouse ABCA7 luciferase construct. After 48 h of transfection, medium was changed to FCS-DF medium plus or minus the latex beads (400 μg/well). The cells were centrifuged for 3 min at 900 *g*, incubated for 1 h, washed, and chased with 10% FCS-DF medium for 6 h. Results represent means ± SD for three samples. *** $P < 0.001$, * $P < 0.05$.

membrane (Fig. 12Da, d), endogenous ABCA7 was localized in the intracellular space (Fig. 12Db, e).

DISCUSSION

In this study, we found that ABCA7 promotes Fc receptor-independent phagocytosis. This result seems consistent with the fact that ABCA7 is highly expressed in reticuloendothelial tissues. CED-7, a similar protein to ABCA1 in *C. elegans*, plays an important role in membrane dynamics and is necessary to mediate actin rearrangement and corpse removal on engulfment with CED-1/CD91/LRP and CED-6/GULP (35). This function was shown to be closely associated with the regulation of this protein through the SREBP2 system. These results were consistent with recent reports indicating that SREBPs are central regulators for membrane biogenesis during phagocytosis (36). CD91/LRP (CED-1 in *C. elegans*) and calreticulin complex, which may recognize an unknown ligand on the apoptotic cell (37), were both increased by phagocytosis of the beads (Fig. 11B).

Fusion of the endoplasmic reticulum with the macrophage plasmalemma underneath phagocytic cups is a source of membrane for phagosome formation in macrophages (38), and endoplasmic reticulum sterol is a regulatory compartment sensed by SREBP/Insig/SCAP (39). Thus, phagocytosis may require membrane biosynthesis and accordingly may induce SREBP maturation for the sterol demand of new membrane. ABCA1 may also promote the engulfment of apoptotic cells and the transbilayer redistribution of phosphatidylserine (40), so the present findings could indicate common functions of ABCA proteins.

We identified a new exon and proximal promoter of the ABCA7 genes. The region contains putative multiple binding motifs for hematopoiesis-related transcription factors such as AML1a, LYF1, MZF1, GATA, and Ikaros2. These factors are highly conserved in mouse and human and are expressed predominantly in the hematopoietic tissues; ABCA7, in fact, is highly expressed in the peripheral leukocytes, thymus, spleen, and bone marrow (9, 13, 17). ABCA7 was also identified as the autoantigen SS-N, an epitope of the autoimmune disease Sjögren's syndrome (41), and ABCA7-positive cells were identified in salivary glands of the patients (42). The ABCA7 gene is juxtaposed to HA-1, which is causatively linked to graft-versus-host disease after allogeneic stem cell transplantation (43), in close proximity on chromosome 19p13.3 (22). Thus, ABCA7 might be an important molecule for hematopoietic development, differentiation, and immunological functions related to lipid homeostasis.

Transcription of the ABCA7 gene is modulated by the cellular sterol level in the opposite direction to that of ABCA1, and this regulation is mediated by the SREBP system, especially by SREBP2. The conclusion is based on the following experimental findings. Sterol depletion induced the upregulation of ABCA7 gene expression (Fig. 1), and this effect disappeared by knockdown of
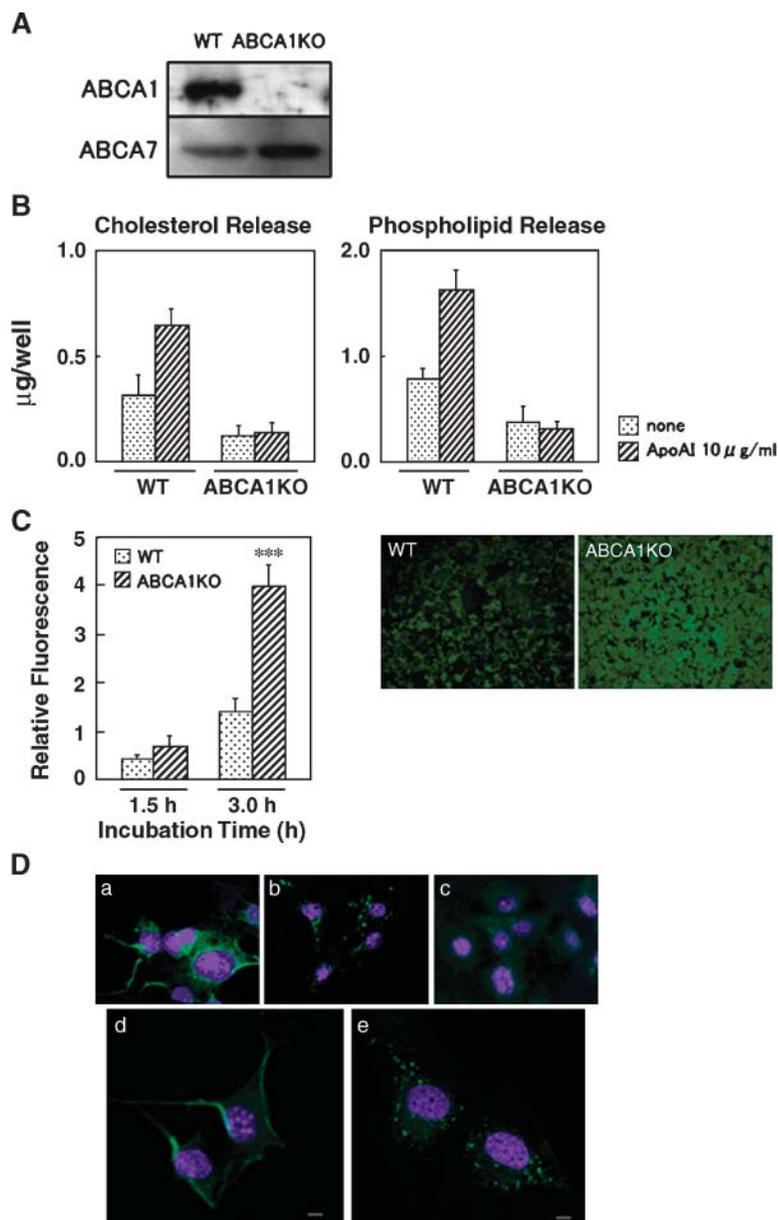
Fig. 12. Analysis of the function of endogenous ABCA7. A: Increase of ABCA7 expression in ABCA1-deficient mouse fibroblasts. Primary lung fibroblasts were cultured on 100 mm plates with 10% FCS-DF medium. Crude membrane fractions (100 μg) were analyzed by immunoblotting for ABCA1 and ABCA7. WT, wild type. B: Cellular lipid release from ABCA1-deficient mouse lung fibroblasts. Cells were subcultured on six-well plates at 70% confluence. After a 48 h incubation, the cells were washed and incubated with serum-free DF medium containing 0.1% BSA with or without 10 μg/ml apoA-I. The conditioned medium was collected after 16 h, and the concentration of lipids was determined. C: Phagocytic activity of ABCA1-deficient mouse fibroblasts. Mouse primary lung fibroblasts were subcultured on 96-well trays at 2.5×10^4/well. After 24 h of incubation, the cells were incubated with 50 μg/well latex beads for the indicated times and washed, and cellular fluorescence intensity was measured. Phagocytosed beads (50 μg/well, 3 h) were imaged by fluorescence microscopy in fibroblasts of each mouse genotype (magnification 4×, green fluorescence). ***$P < 0.001$. D: Subcellular localization of endogenous ABCA1 (a, low magnification; d, high magnification) and ABCA7 (b, low magnification; e, high magnification) in BALB/3T3 cells. c: Cells incubated without specific primary antibody. Fluorescence images were obtained as described in Materials and Methods. Bars = 5 μm. Data represent means ± SD of three samples.

the endogenous SREBPs (Figs. 2C, 6C). Forced expression of SREBP2 upregulated ABCA7 more than SREBP1a (Fig. 2A). ChIP assay (Fig. 7B) and the promoter assay with cotransfected SREBPs showed that the dominant binding factor of SRE is SREBP2 (Figs. 6B, 8C). In addition, SREBP2 increases the ABCA7 reporter activity of plasmid DNA in a dose-dependent manner, but SREBP1a does not (unpublished data).

We previously reported that exogenously introduced ABCA7 mediates the release of cellular lipid and the biogenesis of HDL in vitro, in a similar manner to ABCA1 (8–11). Therefore, it was assumed that the ABCA7 gene is regulated by cellular sterol level similar to the ABCA1 gene, consistent with the previous finding in differentiated macrophages (17). However, we found that ABCA7 is up-

regulated by cellular cholesterol depletion and down-regulated by its increase, the same regulation as seen in the LDL receptor, HMG-CoA reductase, and SREBP2 (44).

In ABCA7-deficient mice, lipid-releasing activity remained normal in macrophage and change in plasma HDL was limited (18). Therefore, ABCA7 may not contribute significantly to the generation of plasma HDL in vivo. Although the in vitro function of ABCA1 and ABCA7 to generate HDL looks similar, it may not be extrapolated to physiological functions of ABCA7 in vivo. Our recent studies suggested fundamental differences between these proteins even for the production of HDL in vitro (10, 11).

Removal of apoptotic cells by phagocytes results in immunosuppression by the production of anti-inflammatory

cytokines and prevents immunoresponse to the internalized and processed proteins of the apoptotic cell debris (45). Thus, autoimmune disorders may be triggered by inefficient clearance of apoptotic cells. This can be related to the finding that ABCA7 is the autoantigen SS-N, an epitope of Sjögren's syndrome (41).

Subcellular localization of ABCA7 was predominantly in the plasma membrane in the ABCA7-expressing HEK293 cells, similar to ABCA1 (8, 9, 12, 13). However, endogenous ABCA7 was localized in the intracellular space in BALB/3T3 cells rather than in the plasma membrane, in agreement with a report on mouse peritoneal macrophages (12). These findings indicate that ABCA7 does not function to export lipid from cells and may rather play an intracellular role consistent with its regulation profile.

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