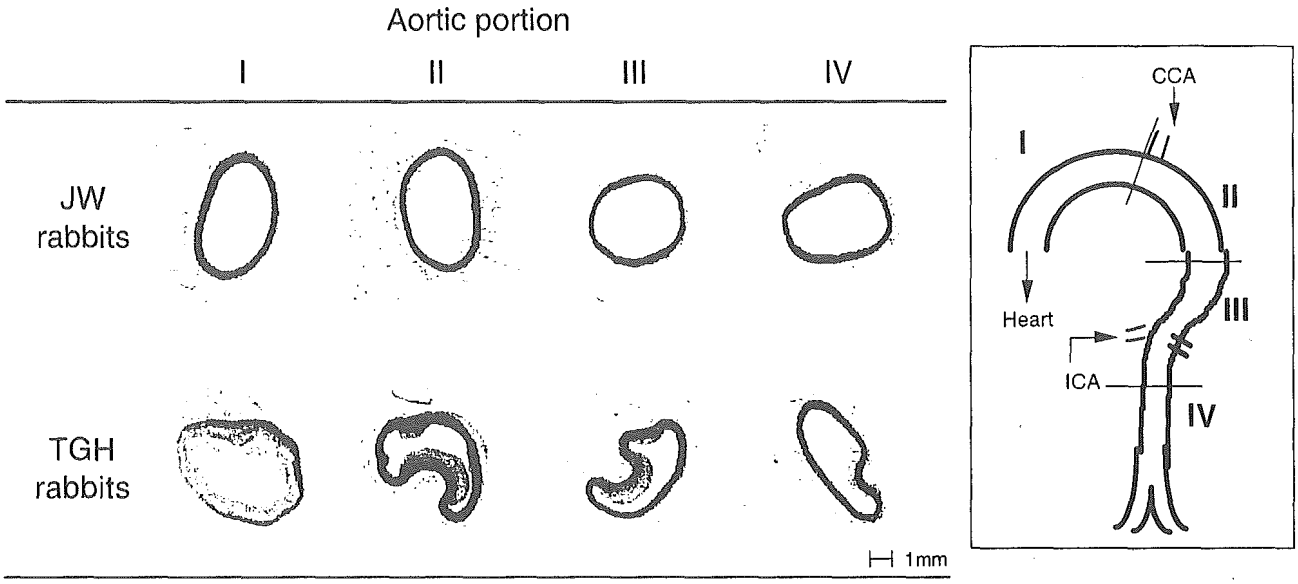

Fig. 1 Representative staining of aortic sections in Japanese white (JW) and TGH (high-triglyceride) rabbits. Portion I, aortic arch; II, proximal thoracic aorta; III, middle thoracic aorta; IV, distal thoracic aorta; CCA, common carotid artery; ICA, intercostal artery.

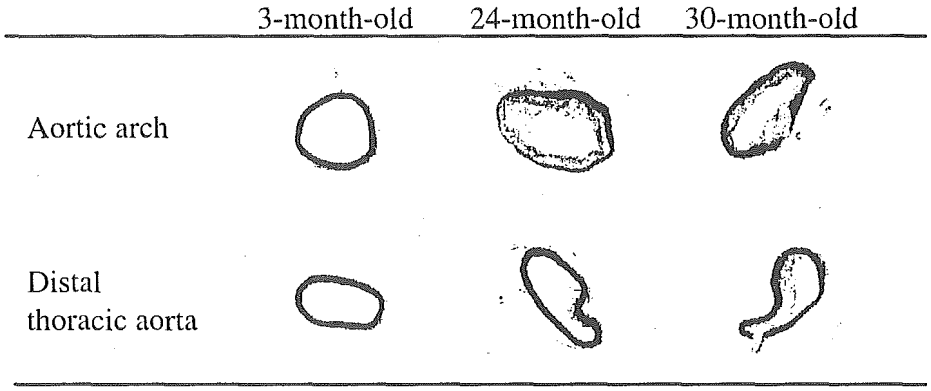

Fig. 2 Age-dependent changes in atherosclerotic lesions in 3-, 24- and 30-month-old TGH rabbits. Representative staining of the aortic arch and distal thoracic aorta.

Table 2 Basal arterial pressure in Japanese white and TGH rabbits

	JW ($n = 5$)	TGH ($n = 6$)
SBP (mmHg)	104.2 ± 8.5	120.8 ± 6.4
DBP (mmHg)	76.2 ± 5.9	72.5 ± 4.5
MAP (mmHg)	85.5 ± 6.3	88.6 ± 4.8
PP (mmHg)	28.0 ± 5.6	48.3 ± 4.5*

Data are the mean±SEM. *$P < 0.01$ compared with Japanese white (JW) rabbits.

TGH, high-triglyceride rabbits; SBP, systolic blood pressure; DBP, diastolic pulse pressure; MAP, mean arterial pressure; PP, pulse pressure.

changes were observed throughout the aortas of TGH rabbits, from the proximal to distal portion. Lesions were most extensive in the aortic arch, whereas atherosclerotic changes seemed to be less in the distal thoracic aorta (Fig. 1). The aortic lesions were characterized by cell proliferation, foam cell accumulation and calcification in the intima. In contrast with the atherosclerosis observed in 24-month-old (young adults for normal rabbits, whose life span is 6–7 years) rabbits, young TGH rabbits (3 months old) did not exhibit atherosclerotic changes in the aorta. In 30-month-old TGH rabbits, the atherosclerotic lesion was considerably extended to the distal thoracic aorta (Fig. 2).

Femoral arterial pressure

Basal arterial pressure

Basal femoral arterial pressure in JW rabbits was 104.2 ± 8.5 mmHg in systole and 76.2 ± 5.9 mmHg in diastole, whereas that in TGH rabbits was 120.8 ± 6.4 mmHg in systole and 72.5 ± 4.5 mmHg in diastole. The systolic pressure in TGH rabbits tended to be higher than that in JW rabbits, but there was no significant difference. However, the pulse pressure was significantly greater in TGH than in JW rabbits (48.3 ± 4.5 *vs* 28.0 ± 5.6 mmHg, respectively; Table 2). The heart rate in JW and TGH rabbits was 195 ± 7 and 185 ± 11 b.p.m., respectively.

Effect of L-NAME

To elucidate whether NO was involved in regulating blood pressure, the effect of L-NAME on femoral arterial blood pressure was examined.

Table 3 Mean arterial pressure and pulse pressure in Japanese white and TGH rabbits after infusion of N^G-nitro-L-arginine methyl ester

	JW ($n = 5$)	TGH ($n = 6$)
SBP (mmHg)	122.8 ± 6.2	141.3 ± 9.8
DBP (mmHg)	91.0 ± 3.7	95.2 ± 5.9
MAP (mmHg)	101.6 ± 2.4	110.6 ± 6.9
% Basal value	121.5 ± 8.4†	125.1 ± 3.5†
PP (mmHg)	31.8 ± 7.9	50.8 ± 3.6*
% Basal value	107.0 ± 10.0	98.0 ± 5.5

Data are the mean±SEM. *$P < 0.01$ compared with Japanese white (JW) rabbits; †$P < 0.05$ compared with basal values.

TGH, high-triglyceride rabbits; SBP, systolic blood pressure; DBP, diastolic pulse pressure; MAP, mean arterial pressure; PP, pulse pressure.

Before infusion of L-NAME, the mean arterial pressure (MAP) in JW and TGH rabbits was 85.5 ± 6.3 and 88.6 ± 4.8 mmHg, respectively. After 10 min, the MAP was 101.6 ± 2.4 and 110.6 ± 6.9 mmHg in JW and TGH rabbits, respectively (Table 3). However, there was no significant difference in the percentage increase in pressure between JW and TGH rabbits (121.5 ± 8.4 and 125.1 ± 3.5%, respectively).

DISCUSSION

In the present study, we investigated haemodynamic parameters in a newly developed rabbit line of heritable hypertriglyceridaemia with hypercholesterolaemia (TGH rabbits) and evaluated the role of mixed hyperlipidaemia in the progression of aortic atherosclerosis histologically.

It is well known that hyperlipidaemia is a major risk factor for atherosclerosis. Epidemiological studies have suggested a relationship between serum TG and the risk of coronary heart disease.[8–10] Recently, it has been demonstrated that hypertriglyceridaemia induces an endothelial dysfunction through increased oxidative stress. The generated superoxide anion can react with NO and produce cytotoxic peroxynitrite.[20] This mechanism may be one possibility for the accelerated atherosclerosis observed in TGH rabbits. In humans, the TG level is an important and independent risk factor for cardiovascular diseases, such as myocardial infarction.[21–25] In addition, an interaction between total cholesterol and TG concentration on the risk of myocardial infarction has been reported.[26,27] Therefore, the new TGH rabbit line may become a useful model of these human diseases associated with hypertriglyceridaemia.

Histopathological changes in the aorta

Of the young rabbits (3 months old), TGH rabbits did not exhibit atherosclerotic changes in the aorta. At 24 months of age (the young-adult age), TGH rabbits showed atherosclerosis and the atherosclerotic lesion was markedly extended to the distal thoracic aorta by 30 months of age.

In JW rabbits, an intact monolayer of endothelial cells covered the aortic lumen and foam cells were not observed in any portion of the aorta examined. In TGH rabbits, endothelial cells were partly detached and fibrous intimal thickening, infiltration of medial smooth muscle cells into the subintima and foam cells were observed. These results suggest that, in TGH rabbits, endothelial cells were damaged and the process of atherosclerosis was proceeding. The production of various cytokines and growth factors by macrophages may further accelerate the migration and growth of medial smooth muscle cells to form atherosclerotic lesions.

The present data show that the degree of atherosclerosis in 24-month-old TGH rabbits is as severe as that in 20–24-month-old WHHL rabbits.[28,29] However, in TGH rabbits, atherosclerotic changes were distributed widely from the aortic arch to the distal thoracic aorta and the pathological changes were most severe in the aortic arch. In contrast, severe histopathological changes tend to occur in the aortic arch and at the bifurcation of arteries in WHHL rabbits.[28] These differences in susceptibility to atherosclerosis may be due to differences in plasma lipids between TGH and WHHL rabbits.

Regulation of basal arterial pressure

Under basal conditions, the systolic and diastolic blood pressures, as well as MAP, of TGH rabbits with severe atherosclerosis were not significantly different those in JW rabbits. These results are consistent with a previous report that the basal blood pressure of WHHL rabbits with atherosclerosis was not higher than that of JW rabbits.[30] Although the blood pressure of TGH rabbits was not significantly different from that of JW rabbits, the pulse pressure of TGH rabbits was significantly greater compared with that of JW rabbits. There is one previous study indicating that the pulse pressure in WHHL rabbits increases gradually with ageing.[31] In addition, 24-month-old Kurosawa and Kusanagi-Hypercholesterolaemic (KHC) rabbits showed greater pulse pressure than 10-month-old KHC rabbits.[32] It should be noted that the increase in pulse pressure observed in these reports may be caused by ageing-related processes rather than hypercholesterolaemia, because elevated plasma cholesterol *per se* may not contribute to the reduced aortic compliance.[33,34] Therefore, the elevated pulse pressure in TGH rabbits compared with that in age-matched JW rabbits in the present study suggests that the elevated pulse pressure may be due to hypertriglyceridaemia or hypertriglyceridaemia with hypercholesterolaemia characterized in TGH rabbits.

It is possible that decreased compliance in atherosclerotic vessels caused the increase in pulse pressure in TGH rabbits, because it has been reported previously that the progression of atherosclerosis reduced compliance in large vessels to increase pulse pressure.[35,36] Pulse pressure arises from the interaction of cardiac stroke volume and the properties of the arterial circulation. In general, increased aortic stiffness of the aorta and large arteries leads to an increase in pulse pressure through a reduction in arterial compliance and effects on wave reflection.[35] Very recently, it has been demonstrated that arterial stiffness is associated with central pulse wave velocity (PWV) and that the PWV correlates well to brachial and central pulse pressure.[37] Therefore, it can be said that central arterial stiffness is closely related to central pulse pressure. Based on these facts, although we did not measure PWV or wall compliance for arterial stiffness directly, the increased pulse pressure in TGH rabbits could be due to arterial stiffness caused by atherosclerosis.

It is quite likely that the elevation of pulse pressure may induce vascular damage and endothelial dysfunction,[36] which can become a possible precursor of atherosclerosis. In fact, it has been found that acetylcholine-induced endothelium-dependent relaxation was decreased in aortas isolated from TGH rabbits.[16] The progression of vascular atherosclerosis and remodelling leads, in turn, to an increase in arterial wall stiffness, thus further amplifying pulse

pressure. Such a vicious cycle probably promotes the progression of atherosclerosis in TGH rabbits to cause many diseases under hyperlipidaemic conditions. Many clinical studies have reported that baseline pulse pressure is a good predictor of subsequent cardiovascular events.[38–40]

Role of NO in the regulation of arterial pressure

Endothelial cells contribute to the maintenance of homeostasis in vascular function and the regulation of systemic circulation via production of NO and endothelial dysfunction is closely related to the progression of atherosclerosis.[6,7] In hyperlipidaemia, the endothelial cell-dependent relaxation of the aorta is impaired, even in early stages without overt histopathological atherosclerotic changes,[3] because lipoproteins, such as oxidized LDL, could injure the endothelial cells to decrease NO production prior to forming atheromatous plaques. In fact, in WHHL rabbits, it has been reported that the plasma concentration of NO is lower than that in JW rabbits[41] and endothelium-dependent vascular relaxation is decreased.[41] It is possible that the decreased basal NO production as a result of endothelial damage may increase the blood pressure through an increase in the tension of vascular walls. Therefore, in the present study, to evaluate the change in basal production of NO in TGH rabbits, we examined the effect of the NOS inhibitor L-NAME on blood pressure. It has been reported previously that N^G-nitro-L-arginine and its methylesterified L-NAME, at 20 mg/kg, increase arterial pressure with decreasing total peripheral conductance in the rabbits.[17] It has been demonstrated that this dose produces maximum blockade of NOS.[17,18] In addition, these NOS blockers were shown to inhibit the hypotensive actions of acetylcholine and the effects were reversed by L-arginine, but not D-arginine, indomethacin or prazosin, demonstrating that the effects were specific to NOS inhibition.[19]

After the addition of L-NAME, systolic blood pressure increased 16 mmHg in JW rabbits and 22 mmHg in TGH rabbits, with no significant difference in responses between JW and TGH rabbits. Therefore, TGH rabbits may produce sufficient NO, as do JW rabbits, although we did not measure the concentration of plasma NO directly in the present study. There are three isoforms of NOS, namely neuronal (n) NOS, inducible (i) NOS (NOS2) and eNOS (NOS3). Of these, nNOS is distributed throughout central and peripheral nerve cells, as well as pancreatic β cells, and is regulated by intracellular calcium, whereas iNOS, which is induced by lipopolysaccarides in hepatic cells, macrophages, intestinal epithelial cells, vascular smooth muscle cells and glial cells, is regulated independently of calcium. Endothelial NOS exists in vascular endothelial cells and its activity is regulated by calcium and the phosphorylation of enzymes.[42] In TGH rabbits, aortic expression of eNOS mRNA and its protein was equivalent to that of JW rabbits, whereas endothelium-dependent vascular relaxation to acetylcholine was reduced in TGH rabbits compared with JW rabbits.[16] These results suggest that the level of eNOS expression is not directly related to NO production in the aorta from TGH rabbits. It could be speculated that iNOS was induced and activated in vascular smooth muscle cells, as well as in macrophages, within atherosclerotic lesions of TGH rabbits to increase the local production of NO. Therefore, it may be important to examine the role of macrophages or vascular smooth muscle cells as a possible source of NO in TGH rabbits. This problem remains to be elucidated.

Genetic background of TGH rabbits

In order to reveal the mode of inheritance of hypertriglyceridaemia, three rabbits with hypertriglyceridaemia were crossed with two wild-type JW rabbits. The F_1 rabbits did not show either hypertriglyceridaemia or hypercholesterolaemia. By intercrossing five pairs of F_1 rabbits, 33 F_2 animals were obtained. Of these, only seven of the 33 rabbits exhibited both hypertriglyceridaemia and hypercholesterolaemia. These results suggest that, in the TGH rabbits, the phenotype of hypertriglyceridaemia follows a single Mendel's law, exhibiting an autosomal recessive inheritance, which is similar to hypercholesterolaemia shown in WHHL rabbits. At present, the main genetic defects in TGH rabbits have not been clarified. Further studies are needed to determine the cause of the elevated triglyceridaemia in this strain.

In summary, the present study has shown that severe atherosclerotic changes developed in TGH rabbits and suggests that hyperlipidaemia with hypercholesterolaemia and hypertriglyceridaemia is an important risk factor for atherosclerosis in TGH rabbits. In addition, the pulse pressure of TGH rabbits was significantly elevated compared with that of normal rabbits. This newly developed TGH rabbit line with heritable hypertriglyceridaemia will become a useful animal model for studies on the role of hyperlipidaemia in the progression of atherosclerosis and in many atherosclerosis-related diseases.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Scientific Research (No. 03COE117 and No. 03COE126), by a Grant-in-Aid for Scientific Research (C) (No. 17590468 to AI and 15590220 to YK) from the Ministry of Education, Science, Sports and Culture, Japan and by grants from The Japan Health Foundation (to AI).

REFERENCES

1. Assmann G, Schulte H, von Eckardstein A, Yadong H. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. *Atherosclerosis* 1996; **124** (Suppl.): S11–20.
2. Shiomi M, Ito T, Hirouchi Y, Enomoto M. Fibromuscular cap composition is important for the stability of established atherosclerotic plaques in mature WHHL rabbits trated with statins. *Atherosclerosis* 2001; **157**: 75–84.
3. Tagawa H, Tomoike H, Nakamura M. Putative mechanisms of the impartment of endothelium-dependent relaxation of the aorta with atheromatous plaque in heritable hyperlipidemic rabbits. *Circ. Res.* 1991; **68**: 330–7.
4. Ross R, Glomset JA. The pathogenesis of atherosclerosis. *N. Engl. J. Med.* 1976; **295**: 377–96.
5. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* 1993; **362**: 801–9.
6. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* 1990; **344**: 160–2.
7. Ooboshi H, Toyoda K, Faraci FM, Lang MG, Heistad DD. Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. *Arterioscler. Thromb. Vasc. Biol.* 1998; **18**: 1752–8.
8. Iso H, Naito Y, Sato S *et al.* Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am. J. Epidemiol.* 2001; **153**: 490–9.

9. Egger M, Smith GD, Pflüger D, Altpeter E, Elwood PC. Triglyceride as a risk factor for ischaemic heart disease in British men: Effect of adjusting for measurement error. *Atherosclerosis* 1999; **143**: 275–84.
10. Rosenson RS. Hypertriglyceridemia and coronary heart disease risk. *Cardiol. Rev.* 1999; **7**: 342–8.
11. Mahley RW, Ji ZS. Remnant lipoprotein metabolism: Key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J. Lipid Res.* 1999; **40**: 1–16.
12. de Man FH, Weverling-Rijnsburger AW, van der Laarse A, Smelt AH, Jukema JW, Blauw GJ. Not acute but chronic hypertriglycèridemia is associated with impaired endothelium-dependent vasodilation: Reversal after lipid-lowering therapy by atorvastatin. *Arterioscler. Thromb. Vasc. Biol.* 2000; **20**: 744–50.
13. Inoue T, Saniabadi AR, Matsunaga R, Hoshi K, Yaguchi I, Morooka S. Impaired endothelium-dependent acetylcholine-induced coronary artery relaxation in patients with high serum remnant lipoprotein particles. *Atherosclerosis* 1998; **139**: 363–7.
14. Lundman P, Tornvall P, Nilsson L, Pernow J. A triglyceride-rich fat emulsion and free fatty acids but not very low density lipoproteins impair endothelium-dependent vasorelaxation. *Atherosclerosis* 2001; **159**: 35–41.
15. Takasaki S, Zhang C, Ito T, Tomoike H. Does association of hypercholesterolemia and hypertriglyceridemia augment aortic atherosclerosis? *Circulation* 1999; **100** (Suppl.): I–698 (Abstract).
16. Shishido T, Tasaki K, Takeishi Y *et al.* Chronic hypertriglyceridemia in young watanabe heritable hyperlipidemic rabbits impairs endothelial and medial smooth function. *Life Sci.* 2004; **74**: 1487–501.
17. Brooks VL, Clow KA, Welch LS, Giraud GD. Does nitric oxide contribute to the basal vasodilation of pregnancy in consciour rabbits? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001; **281**: R1624–32.
18. Miller SL, Jenkin G, Walker DW. Effect of nitric oxide synthase inhibition on the uterine vasculature of the late-pregnant ewe. *Am. J. Obstet. Gynecol.* 1999; **180**: 1138–45.
19. Rees DD, Palmer RMJ, Moncada S. Role of endothelum-derived nitric oxide in the regulation of blood pressure. *Proc. Natl Acad. Sci. USA* 1989; **86**: 3375–8.
20. Bae JH, Bassenge E, Kim KB *et al.* Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. *Atherosclerosis* 2001; **155**: 517–23.
21. Austin MA, Hokanson JE, Edwards KL. Hypertriglyceridemia as a cardiovascular risk factor. *Am. J. Cardiol.* 1998; **81** (Suppl. 1): B7–12.
22. Patsch JR, Miesenbock G, Hopferwieser T *et al.* Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. *Arterioscler. Thromb. Vasc. Biol.* 1992; **12**: 1336–45.
23. Stampfer MJ, Krauss RM, Ma J *et al.* A prospective study of triglyceride level, low density lipoprotein particle diameter, and risk of myocardial infarction. *JAMA* 1996; **276**: 882–8.
24. Jeppesen J, Hein HO, Suadicani P, Gyntelberg F. Triglyceride concentration and ischemic heart disease: An eight-year follow-up in the Copenhagen Male Study. *Circulation* 1998; **97**: 1029–36.
25. Karpe F, Steiner G, Uffelman K, Olivecrona T, Hamsten A. Postprandial lipoproteins and progression of coronary atherosclerosis. *Atherosclerosis* 1994; **106**: 83–97.
26. Stavenow L, Kjellstrom T. Influence of serum triglyceride levels on the risk for myocardial infarction in 12 510 middle aged males: Interaction with serum cholesterol. *Atherosclerosis* 1999; **147**: 243–7.
27. Gotto AM. Triglyceride: The forgotten risk factor. *Circulation* 1998; **97**: 1027–8.
28. Shiomi M, Ito T, Tsukada T, Yata T, Ueda M. Cell compositions of coronary and aortic atherosclerotic lesions in WHHL rabbits differ. *Arterioscler. Thromb.* 1994; **14**: 931–7.
29. Murakami S, Kondo Y, Sakurai T, Kitajima H, Nagate T. Taurine suppresses development of atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits. *Atherosclerosis* 2002; **163**: 79–87.
30. Yamada S, Ito T, Adachi J, Ueno Y, Shiomi M. Decreased arterial responses in WHHL rabbits, an animal model of spontaneous hypercholesterolemia and atherosclerosis. *Exp. Anim.* 2002; **51**: 493–9.
31. Hasegawa M, Watanabe Y. Rheological properties of the thoracic aorta in normal and WHHL rabbits. *Biorheology* 1988; **25**: 147–56.
32. Katsuda S, Hasegawa M, Kusanagi M, Shimizu T. Comparison of pulse-wave velocity in different aortic regions in relation to the extent and severity of atherosclerosis between young and older Kurosawa and Kusanagi-Hypercholesterolemic (KHC) rabbits. *Clin. Sci.* 2000; **99**: 393–404.
33. Lehmann EW, Watts GF, Gosling GS. Aortic distensibility and hypercholesterolemia. *Lancet* 1991; **338**: 270–3.
34. Pitsavos C, Toutouzas K, Dernellis J *et al.* Aortic stiffness in young patients with heterozygous familial hypercholesterolemia. *Am. Heart J.* 1998; **135**: 604–8.
35. Dart AM, Kingwell BA. Pulse pressure: A review of mechanisms and clinical relevance. *J. Am. Coll. Cardiol.* 2001; **37**: 975–84.
36. Ryan SM, Waack BJ, Weno BL, Heistad DD. Increases in pulse pressure impair acetylcholine-induced vascular relaxation. *Am. J. Physiol.* 1995; **268**: H359–63.
37. Woodman RJ, Kingwell BA, Beilin LJ, Hamilton SE, Dart AM, Watts GF. Assessment of central and peripheral arterial stiffness: Studies indicating the need to use a combination of techniques. *Am. J. Hypertens.* 2005; **18**: 249–60.
38. Lee ML, Rosner BA, Weiss ST. Relationship of blood pressure to cardiovascular death: The effects of pulse pressure in the elderly. *Ann. Epidemiol.* 1999; **9**: 101–7.
39. Verdecchia P, Schillaci G, Borgioni C, Ciucci A, Pede S, Porcellati C. Ambulatory pulse pressure: A potent predictor of total cardiovascular risk in hypertension. *Hypertension* 1998; **32**: 983–8.
40. Benetos A, Safar M, Rudnichi A *et al.* Pulse pressure: A predictor of long-term cardiovascular mortality in a French male population. *Hypertension* 1997; **30**: 1410–15.
41. Kanazawa K, Kawashima S, Mikami S *et al.* Endothelial Constitutive nitric oxide synthase protein and mRNA increased in rabbit atherosclerotic aorta despite impaired enrothelium-dependent vascular relaxation. *Am. J. Pathol.* 1996; **148**: 1949–56.
42. Furchugott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. *FASEB J.* 1989; **3**: 2007–18.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 10, Issue of March 7, pp. 8815–8819, 2003
Printed in U.S.A.

Effects of Mutations of ABCA1 in the First Extracellular Domain on Subcellular Trafficking and ATP Binding/Hydrolysis*[S]

Received for publication, July 10, 2002, and in revised form, December 5, 2002
Published, JBC Papers in Press, December 31, 2002, DOI 10.1074/jbc.M206885200

Arowu R. Tanaka‡§¶, Sumiko Abe-Dohmae¶‖, Tomohiro Ohnishi‡, Ryo Aoki‡, Gaku Morinaga‡, Kei-ichiro Okuhira‖, Yuika Ikeda‡, Fumi Kano¶, Michinori Matsuo‡, Noriyuki Kioka‡, Teruo Amachi‡, Masayuki Murata¶, Shinji Yokoyama‖, and Kazumitsu Ueda‡**

From the ‡Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, ‖Biochemistry, Cell Biology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan, and the ¶Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444-8585, Japan

ABCA1 mediates release of cellular cholesterol and phospholipid to form high density lipoprotein (HDL). The three different mutants in the first extracellular domain of human ABCA1 associated with Tangier disease, R587W, W590S, and Q597R, were examined for their subcellular localization and function by using ABCA1-GFP fusion protein stably expressed in HEK293 cells. ABCA1-GFP expressed in HEK293 was fully functional for apoA-I-mediated HDL assembly. Immunostaining and confocal microscopic analyses demonstrated that ABCA1-GFP was mainly localized to the plasma membrane (PM) but also substantially in intracellular compartments. All three mutant ABCA1-GFPs showed no or little apoA-I-mediated HDL assembly. R587W and Q597R were associated with impaired processing of oligosaccharide from high mannose type to complex type and failed to be localized to the PM, whereas W590S did not show such dysfunctions. Vanadate-induced nucleotide trapping was examined to elucidate the mechanism for the dysfunction in the W590S mutant. Photoaffinity labeling of W590S with 8-azido-[α-^{32}P]ATP was stimulated by adding *ortho*-vanadate in the presence of Mn^{2+} as much as in the presence of wild-type ABCA1. These results suggest that the defect of HDL assembly in R587W and Q597R is due to the impaired localization to the PM, whereas W590S has a functional defect other than the initial ATP binding and hydrolysis.

Cholesterol is not catabolized in the peripheral cells and therefore mostly released and transported to the liver for conversion to bile acids to maintain cholesterol homeostasis. The same pathway may also remove cholesterol that has pathologically accumulated in the cells such as an initial stage of atherosclerosis. Assembly of high density lipoprotein (HDL)[1] particles by helical apolipoproteins with cellular lipid has been recognized as one of the major mechanisms for cellular cholesterol release (1, 2). The importance of this active cholesterol-releasing pathway in regulating cholesterol homeostasis became apparent by the finding that it is impaired in the cells from patients with Tangier disease, a genetic deficiency of circulating HDL (3, 4). Mutations were identified in ATP-binding cassette transporter A1 (ABCA1) of the Tangier disease (TD) patients (5–7), but the molecular mechanism of ABCA1 in the apolipoprotein-mediated HDL assembly remains unclear. Although direct interaction between ABCA1 and apoA-I at the cell surface has been suggested on the basis of chemical cross-linking experiments (8, 9), an indirect role of ABCA1 in the apoA-I binding to the cell was also proposed by a model that ABCA1 induces phosphatidylserine exofacial flopping to generate the microenvironment required for the docking of apoA-I at the cell surface (10). The predominant substrates of the ABCA1-mediated lipid release reaction are still to be determined for the HDL assembly reaction (11, 12).

More than 30 mutations have been mapped in the *ABCA1* gene in patients with familial hypoalphalipoproteinemia (FHA) and TD (5–7, 13–15). Many mutations have been identified in the putative first extracellular domain (ECD1) and the first nucleotide binding fold (NBF1) of ABCA1. We and Fitzgerald *et al.* (16–18) recently demonstrated that ECD1 exists in the extracellular space by introducing an epitope tag into ABCA1 ECD1 and by analyzing glycosylation of the truncated form of ABCA1. To investigate the mechanistic background for these mutations to cause the dysfunction of ABCA1, we characterized the function and subcellular localization of ABCA1-GFP and its TD mutants stably expressed in HEK293 cells. Three TD mutants (R587W, W590S, Q597R), clustered in ECD1, were examined in the present report. Immunostaining and confocal microscopic analysis showed that ABCA1 is mainly localized to the plasma membrane (PM), where ECD1 is expected to be exposed to the outside of the cell, but also in intracellular compartments to a substantial extent. The TD mutations in ECD1 resulted in a distinct influence on the function and subcellular localization of ABCA1. All three mutants were functionally impaired for the apoA-I-mediated HDL assembly. On the other hand, the two mutants R587W and Q597R were only partially or scarcely localized to the PM, whereas W590S

* This work was supported by Grant-in-aid for Scientific Research 10217205 on Priority Areas "ABC Proteins" from the Ministry of Education, Science, Sports, and Culture of Japan and by the Nakajima Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplementary data showing a figure showing the trafficking of ABCA1-GFP in HEK293 cells and a figure showing the characterization of ABCA1-GFP vesicles.

¶ These authors contributed equally to the work.

** To whom correspondence should be addressed. Tel.: 81-75-753-6105; Fax: 81-75-753-6104; E-mail: uedak@kais.kyoto-u.ac.jp.

[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; ECD1, the first extracellular domain; NBF, nucleotide binding fold; TD, Tangier disease; FHA, familial hypoalphalipoproteinemia; PM, plasma membrane; ER, endoplasmic reticulum; Endo H, endoglycosidase H; PNGaseF, *N*-glycosidase F; GFP, green fluorescent protein.

was localized to the PM as efficiently as the wild type. Vanadate-induced nucleotide trapping was examined to elucidate the mechanism for the dysfunction in the W590S mutant.

EXPERIMENTAL PROCEDURES

Materials—Anti-GFP antibody was purchased from Santa Cruz Biotechnology. All other chemicals were obtained from Sigma, Wako Pure Chemical Industries, or Nacalai Tesque.

Generation of an Antibody to ABCA1 ECD1—The putative extracellular domain ECD1, amino acids 45–639 of human ABCA1, was expressed as a C terminus His tag fusion protein in *Escherichia coli* BL21(DE3) and purified by Ni^{2+} chromatography (Qiagen). A rat polyclonal antibody, generated using this His tag-fused ECD1, specifically interacted with human ABCA1 stably or transiently expressed in HEK293 cells in Western blotting (data not shown) and immunostaining (see Fig. 1).

Immunostaining and Fluorescence Microscopy—Cells were grown on a 35-mm glass-base dish (Iwaki). The cells were incubated with primary antibodies in phosphate-buffered saline containing 5% skim milk. After being washed, these cells were incubated with the fluorescent-labeled secondary antibodies. The cells were directly viewed with ×100 Plan-NEOFLUAR oil immersion objective on a Zeiss confocal microscope LSM510.

DNA Construction—DNA fragments (*Xho*I-*Bcl*I) containing each missense TD mutation (R587W, W590S, or Q597R) were generated using the polymerase chain reaction method with R587W (*Xho*I) primer (5′-GTCCTCGAGCTGACCCCTTTGAGGACATGTGGTACGTC-3′), W590S (*Xho*I) primer (5′-GTCCTCGAGCTGACCCCTTTGAGGACATGCGGTACGTCTCGGGGGGCTTC-3′), or Q597 (*Xho*I) primer (5′-GTCCTCGAGCTGACCCCTTTGAGGACATGCGGTACGTCTGGGGGGGCTTCGCCTACTTGCGGGATGTGGTG-3′), where the mutated nucleotide is underlined, and *Bcl*I primer (5′-CGATGCCCTTGATGATCACAGCCACTGAG-3′). The DNA fragment was replaced with the *Xho*I-BclI fragment of human ABCA1 (16).

Glycosylation of ABCA1-GFP Protein—Endoglycosidase H (Endo H) and peptide *N*-glycosidase F (PNGaseF) (New England Biolabs, Beverly, MA) digestions were done as described by the manufacturer. In brief, 10 μg of membrane proteins from HEK293 cells stably expressing the wild-type, R587W, W590S, or Q597R ABCA1-GFP were treated with 500 units of Endo H or 0.3 units of PNGaseF for 1 h at 37 °C. The deglycosylated proteins were separated by SDS-PAGE (7.5%) and analyzed by immunoblotting by using the anti-GFP antibody.

Cellular Lipid Release Assay—Cells were subcultured in 6-well plates (TPP, 92406) at a density of 1.0×10^6 cells in a 1/1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) supplemented with 10% (v/v) fetal calf serum. After incubation for 48 h, the cells were washed with Dulbecco's phosphate-buffered saline and incubated in the same medium supplemented with 0.1% bovine serum albumin and 10 μg/ml apoA-I. The lipid content in the medium was determined after a 24-h incubation as described previously (19). To compare lipid release from HEK293 cells transiently expressing ABCA1-GFP, GFP fluorescence of transfected cells was measured with a FL600 fluorescent plate reader (Bio-Tek Instruments, Inc.) (19), and expression levels of the wild-type and mutant ABCA1-GFP were normalized with GFP fluorescence. Expression levels of the wild-type and mutant ABCA1-GFP were in a range of ±20%.

Vanadate-induced Nucleotide Trapping in ABCA1 with 8-Azido-[α-^{32}P]ATP—A membrane fraction (20–30 μg) was prepared from HEK293 cells stably expressing the wild-type or W590S ABCA1-GFP. It was incubated with 15 μM 8-azido-[α-^{32}P]ATP, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-Cl, pH 7.5, in a total volume of 6 μl for 15 min at 37 °C in the presence or absence of 1 mM *ortho*-vanadate and 3 mM $MgSO_4$ or $MnCl_2$. The reaction was stopped by adding 500 μl of ice-cold 40 mM Tris-Cl buffer containing 0.1 mM EGTA and 1 mM $MgSO_4$ or $MnCl_2$. The supernatant containing unbound ATP was removed from the membrane pellet after centrifugation (15,000 × *g*, 5 min, 2 °C), and this procedure was repeated once more. The pellets were resuspended in 8 μl of TE buffer containing 1 mM $MgSO_4$ or $MnCl_2$ and irradiated for 5 min (at 254 nm, 8.2 mW/cm^2) on ice. The sample was analyzed by autoradiogram after electrophoresis in a 7% SDS-polyacrylamide gel. Experiments were done in triplicate.

RESULTS

Subcelluar Localization of ABCA1-GFP—In our previous report, we have shown that the hemagglutinin epitope inserted between residues 207 and 208 of human ABCA1 was recognized by the anti-hemagglutinin antibody from the outside of cells (16). To confirm the extracellular localization of the hydrophilic domain containing residue 207 (ECD1), non-permeabilized HEK293 cells were incubated with a rat polyclonal antibody against the protein corresponding to amino acids 45–639 of human ABCA1 for immunostaining. ABCA1-GFP, which was apparently on the cell surface, was visualized by the antibody, whereas the protein in the intracellular compartments was not (Fig. 1). The results suggested that ABCA1-GFP was localized to the PM and the intracellular compartments, and the ECD1 domain of ABCA1 on the PM was exposed to outside of the cells.

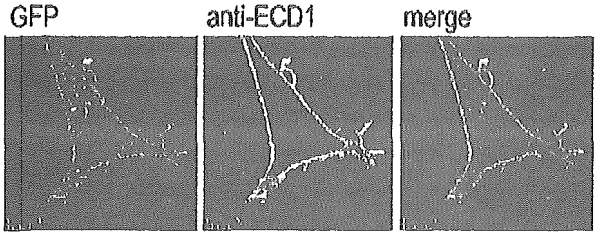

FIG. 1. **Immunofluorescence confocal microscopy analysis of HEK293 cells stably expressing ABCA1-GFP.** *GFP*, GFP fluorescence of HEK293 cell stably expressing ABCA1-GFP. *Anti-ECD1*, immunofluorescent observation with anti-ECD1 antibody and anti-rat IgG-Alexa594. *Merge*, overlaid GFP and Alexa594 fluorescence.

Effects of ECD1 Mutations on Subcellular Localization of ABCA1-GFP—Many mutations in patients with TD and FHA have been identified in ECD1 of ABCA1, and three mutations (R587W, W590S, Q597R) cluster in the vicinity between amino acids 587 and 597 (20)(Fig. 2*A*). To study the role of ECD1 domain in the HDL assembly function of ABCA1, we introduced these three TD mutations in ECD1 into ABCA1-GFP and transiently or stably expressed in HEK293. The expression levels of mutant ABCA1-GFP stably expressed in HEK293 cells were comparable with that of the wild-type ABCA1-GFP as shown later in Figs. 3 and 5. Confocal microscopic examination revealed that R587W and Q597R appeared to be localized mainly in the ER and not to the PM (Fig. 2*B*). In contrast, W590S was localized to the PM as much as the wild-type ABCA1-GFP was, although more was found with intracellular vesicles than with the wild type (Fig. 2*B*). Immunostaining with the antibody against ECD1 confirmed the proper orientation of W590S (Fig. 2*C*).

Glycosylation of ABCA1-GFP—Glycosylation of the wild-type ABCA1-GFP and its mutants R587W, W590S, and Q597R was examined by the treatment with PNGaseF and Endo H (Fig. 3*A*). Endo H cleaves two proximal *N*-acetylglucosamine residues of the high mannose type but not of the complex type, whereas PNGaseF cleaves sugar chains of both the high mannose and complex types. The treatment with PNGaseF increased the electrophoretic mobilities of 280-kDa ABCA1 to produce the 250-kDa protein, the deglycosylated form of ABCA1-GFP. ABCA1 with TD mutations, R587W and Q597R ABCA1-GFP, was sensitive to Endo H to produce the deglycosylated form of ABCA1-GFP, whereas the wild-type ABCA1-GFP was little digested by Endo H. These results indicated that R587W and Q597R ABCA1-GFP did not contain complex oligosaccharides and supported the confocal microscopy observation, which suggested the localization of these two TD mutants in the ER or the cis-Golgi complex. On the other hand, W590S ABCA1-GFP was resistant to Endo H, indicating that it does not contain high mannose oligosaccharides but contains complex oligosaccharides and reached the trans-Golgi complex.

Effects of ECD1 Mutations on apoA-I-mediated Cholesterol Release—To analyze the functional consequences of these mutations, apoA-I-mediated release of cholesterol and choline-

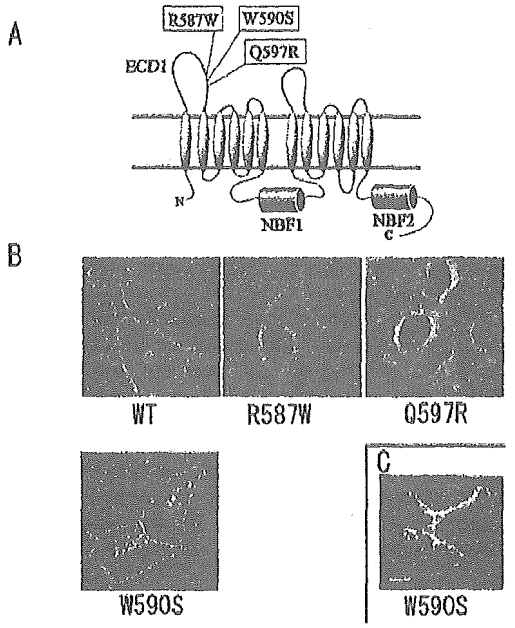

FIG. 2. **Effects of ECD1 mutations on subcellular localization of ABCA1-GFP.** *A*, a putative secondary structure of ABCA1 and localization of Tangier Disease mutations R587W, W590S, and Q597R in ECD1. *B*, GFP fluorescence of HEK293 cells stably expressing the wild-type (*WT*) ABCA1-GFP and three TD mutants R587W, W590S, and Q597R ABCA1-GFP. *C*, immunofluorescent observation of W590S ABCA1-GFP with anti-ECD1 antibody and anti-rat IgG-Alexa594.

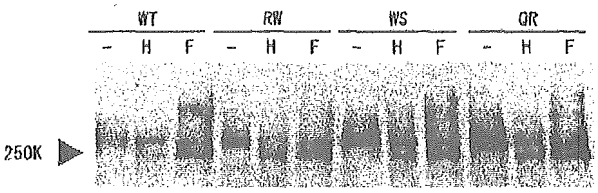

FIG. 3. **Glycosylation of ABCA1-GFP.** The wild-type (*WT*), R587W, W590S, and Q597R ABCA1-GFP were treated with none (−), Endo H (*H*), or PNGaseF (*F*) and separated with 7% SDS-PAGE. Western blotting was done with anti-GFP antibody.

phospholipid was examined from the stable transformants (Fig. 4, *A* and *B*). The wild-type ABCA1-GFP exported 0.14 ± 0.01 and 1.14 ± 0.07 μg of cholesterol from cells grown in 6-well plates in the absence and presence of apoA-I, respectively, and 1.99 ± 0.25 and 5.88 ± 0.13 μg of choline-phospholipids, respectively, to generate HDL in the medium by reducing about 15% of cell cholesterol. The R587W mutation resulted in the apoA-I-mediated release of cholesterol and choline-phospholipids to 24 and 23% of the wild-type ABCA1-GFP, respectively. The Q597R mutant almost completely lost this activity. The results were apparently consistent with the inefficient localization to the cell surface of ABCA1-GFP with these mutations. The apoA-I-mediated release of cholesterol and choline-phospholipids from HEK293 expressing W590S ABCA1-GFP were 7.4 and 13%, respectively, of those expressing the wild-type ABCA1-GFP, although W590S ABCA1-GFP was localized to the PM as efficiently as the wild type. The apoA-I-mediated release of cellular cholesterol and choline-phospholipids was also examined with HEK293 transiently expressing the wild-type and mutant ABCA1-GFPs (Fig. 4, *C* and *D*). The results were similar to those observed with the stable transformants shown in Fig. 4, *A* and *B*.

Interaction of ABCA1-GFP with 8-Azido-[α-^{32}P]ATP—To elucidate the mechanism for the loss of function of ABCA1 in the W590S mutant, we examined the interaction of ABCA1-GFP with ATP. Among membrane proteins of the cells expressing the wild-type ABCA1-GFP, a 280-kDa protein was specifically photoaffinity-labeled with ATP (Fig. 5*A*, *lane 7*), whereas no protein was labeled in the untransfected HEK293 cells (*lane 5*). The mobility of the photoaffinity-labeled membrane protein in SDS-PAGE was identical to that of the wild-type ABCA1-GFP visualized by Western blotting (Fig. 5*B*, *lane 1*). The photoaffinity labeling was negative when the samples were incubated in the absence of Mg^{2+} (Fig. 5*A*, *lane 3*), indicating that 8-azido-[α-^{32}P] ATP tightly binds to ABCA1 in the presence of Mg^{2+}.

Multidrug transporters, MDR1 (ABCB1), MRP1 (ABCC1), and MRP2 (ABCC2), are known to trap Mg-ADP in the presence of *ortho*-vanadate, an analog of phosphate, and form a stable inhibitory intermediate during the ATP hydrolysis cycle. Photoaffinity labeling of these proteins with 8-azido-[α-^{32}P] ATP is therefore stimulated when the membrane containing these proteins reacts with the nucleotide in the presence of *ortho*-vanadate (21–24). We thus expected that *ortho*-vanadate would stimulate photoaffinity labeling of ABCA1. However, no increase of photoaffinity labeling of ABCA1 was observed (*lane 8*) in comparison with that in the absence of *ortho*-vanadate (*lane 7*).

Vanadate did not induce nucleotide trapping in MRP6 (ABCC6) in the presence of Mg^{2+}, but it did with Ni^{2+} ions (25). Therefore, we examined photoaffinity labeling of ABCA1-GFP in the presence of other metal ions. Significant stimulation was observed with wild-type ABCA1-GFP by *ortho*-vanadate in the presence of Mn^{2+} (Fig. 5*A*, *lanes 11* and *12*). These results suggested that Mn-ATP was hydrolyzed at NBFs of ABCA1, and a stable inhibitory complex ABCA1-MnADP-Vi was formed

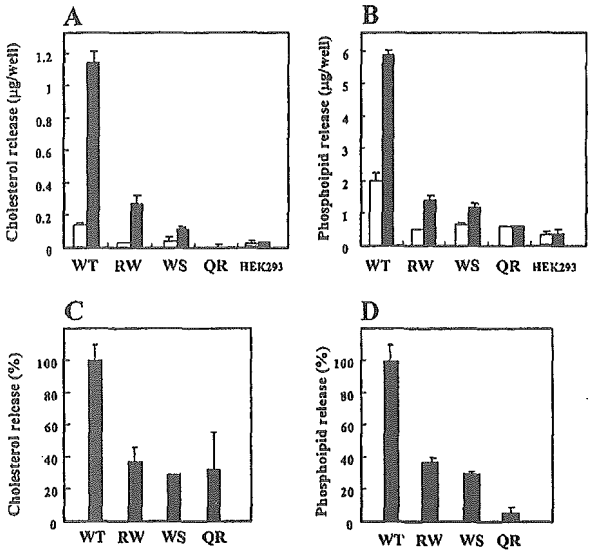

FIG. 4. **Effects of ECD1 mutations on apoA-I-mediated cholesterol and phospholipid transport.** Cholesterol (*A*) and choline-phospholipid (*B*) content in the medium in a 6-well plate containing HEK293 cells stably expressing the wild-type (*WT*), R587W (*RW*), W590S (*WS*), and Q597R (*QR*) ABCA1-GFP were measured after a 24-h incubation in the presence (*black bars*) or absence (*white bars*) of 10 μg/ml apoA-I. The relative amount of cholesterol (*C*) and choline-phospholipid (*D*) in the medium in a 6-well plate containing HEK293 cells transiently expressing the wild-type (*WT*), R587W (*RW*), W590S (*WS*), and Q597R (*QR*) ABCA1-GFP was measured after a 24-h incubation in the presence of 10 μg/ml apoA-I. The expression levels of mutants were normalized with the GFP fluorescence of cells. Lipid release from HEK293 cells transiently expressing ABCA1-GFP subtracted by that from non-transformed HEK293 cells was represented as 100% in *C* and *D*.

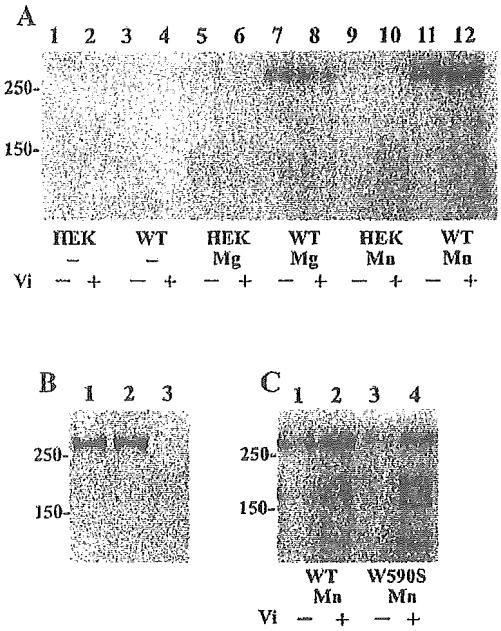

FIG. 5. **Vanadate-induced trapping in ABCA1-GFP.** *A*, photoaffinity labeling of ABCA1-GFP with 8-azido-[α-^{32}P]ATP. Membranes (20 μg) prepared from HEK293 cells stably expressing the wild-type (*WT*) ABCA1-GFP (*lanes 3, 4, 7, 8, 11,* and *12*) or from untransfected HEK293 cells (*HEK*) (*lanes 1, 2, 5, 6, 9,* and *10*) were incubated with 15 μM 8-azido-[α-^{32}P]ATP in the absence or presence of 1 mM *ortho*-vanadate (*Vi*) and 3 mM $MgSO_4$ (*lanes 5–8*) or $MnCl_2$ (*lanes 9–12*) for 15 min at 37 °C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under "Experimental Procedures." *B*, immunoblots of membranes prepared from HEK293 cells stably expressing the wild-type (4 μg, *lane 1*) or W590S (6 μg, *lane 2*) ABCA1-GFP or from untransfected HEK293 cells (6 μg, *lane 3*). Proteins were separated on a 7% SDS-polyacrylamide gel and reacted with monoclonal antibody against GFP. *C*, membranes prepared from HEK293 cells stably expressing the wild type (*WT*) (20 μg, *lanes 1* and *2*) or W590S (30 μg, *lanes 3* and *4*) were incubated with 15 μM 8-azido-[α-^{32}P]ATP in the absence or presence of 1 mM *ortho*-vanadate (*Vi*) and 3 mM $MnCl_2$ for 15 min at 37 °C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under "Experimental Procedures."

during the ATP hydrolysis cycle.

To determine whether the W590S mutation affects the ATP hydrolysis cycle of ABCA1, vanadate-induced nucleotide trapping in W590S ABCA1-GFP was examined in the presence of Mn^{2+} (Fig. 5*C*). Membrane proteins from HEK293 cells expressing a similar amount of wild-type ABCA1 or W590S ABCA1-GFP (Fig. 5*B*) were incubated with 8-azido-[α-^{32}P]ATP in the absence or presence of *ortho*-vanadate. The photoaffinity labeling of W590S ABCA1-GFP was stimulated by adding *ortho*-vanadate in the presence of Mn^{2+} as much as in the presence of the wild type.

DISCUSSION

In this work, we described the influence of three clustered mutations in ECD1 associated with TD and FHA on the subcellular localization of ABCA1, apoA-I-mediated HDL assembly, apoA-I binding, and vanadate-induced nucleotide trapping. Immunostaining of ABCA1-GFP stably expressed in HEK293 cells revealed that ABCA1-GFP apparently resided on the cell surface as well as in intracellular compartments in agreement with previous reports (18, 26–28). Although the three mutations all reduced apoA-I-mediated lipid release and subsequent HDL assembly from HEK293 cells expressing ABCA1-GFP, whether transiently or stably, the mutants demonstrated differential behavior with respect to their subcellular localization. ABCA1-GFP with a R587W or Q597R mutation appeared to be impaired with intracellular trafficking and predominantly localized in the ER. On the other hand, W590S ABCA1-GFP was mainly localized to the PM as much as the wild-type ABCA1 was. The sensitivity to Endo H of the mutant ABCA1s was consistent with the their apparent impairment of intracellular trafficking. R587W and Q597 ABCA1-GFP contained high mannose oligosaccharides, indicating that they do not reach the trans-Golgi complex. In contrast, W590S ABCA1-GFP contained complex-type oligosaccharides as the wild-type does.

When the cells were treated with monensin, which prevents the delivery of protein from endosomes to the cell surface, after inhibiting protein synthesis by treatment with cycloheximide, ABCA1-GFP on the cell surface decreased, and the vesicular localization increased instead (see supplementary data, Fig. 1).[2] When the cells were treated with brefeldin A, which blocks vesicular transport from the ER to the Golgi and to the cell surface along with the secretary pathway (29), the newly synthesized ABCA1-GFP was accumulated in the fused Golgi-ER, the amount of ABCA1-GFP on the PM was reduced, and the vesicles containing ABCA1-GFP were observed (see supplementary data 1).[2] ABCA1-GFP was co-localized partly with Vti1b, a marker for the Golgi, with EEA1, a marker for early endosomes, and with lysotracker, a marker for acidic compartments (see supplementary data 2).[2] These results suggested that newly synthesized ABCA1-GFP was first delivered to the PM through the ER and the Golgi and then shuttled rapidly between the PM and the intracellular vesicles, mainly the early endosomes. R587W and Q597R ABCA1-GFP appeared to be retained in the ER. It has been reported that one amino acid (Phe-508) deletion of the cystic fibrosis transmembrane conductance regulator (CFTR), the major mutation in cystic fibrosis patients that causes misfolding of the cystic fibrosis transmembrane conductance regulator, is degraded by the proteasome pathway before exiting from the ER (30). This region (R587 to Q597) in ECD1 would be critical for proper folding of ABCA1 and would probably affect the intracellular translocation process, whereas the W590S mutation does not. Fitzgerald *et al.* (17) reported that R587W or Q597R mutation did not affect the PM localization but disrupted the direct interaction with ApoA-I. This supports a major conformational alteration of ECD1 by these mutations. The reason for the discrepancy of subcellular localization is unknown between their results with the mutant ABCA1 transiently expressed in a high amount in HEK293 and ours studied with the mutant ABCA1-GFP in the stable transformants with modest expression.

W590S ABCA1-GFP was localized to the PM as much as the wild-type ABCA1-GFP when expressed in HEK293. However, apoA-I-mediated release of cellular cholesterol and choline-phospholipid was severely impaired. To elucidate the reason for this functional impairment in the W590S mutant, nucleotide interaction was examined with the wild-type and W590S ABCA1-GFP by using 8-azido-[α-^{32}P]ATP. The wild-type ABCA1-GFP was photoaffinity-labeled in the presence of Mg^{2+}, but no vanadate-induced nucleotide trapping was observed, being consistent with human ABCA1 expressed in Sf9 insect cells (31). Other transporter-type ABC proteins, such as MDR1 (ABCB1), MRP1 (ABCC1), and MRP2 (ABCC2), trap Mg-ADP in the presence of *ortho*-vanadate and form a stable inhibitory intermediate during the ATP hydrolysis cycle (21–24) so that ABCA1, showing no obvious vanadate-induced nucleotide trapping, was proposed not to be an active transporter but a regulator in apoA-I-dependent cholesterol release (31). However, vanadate-induced nucleotide trapping was demon-

strated to be positive with ABCA1 in the presence of Mn^{2+} in this study. Vanadate-induced nucleotide trapping did not occur in the presence of Mg^{2+} but can be detected with Ni^{2+} ions in MRP6 (ABCC6) (25), and it was detected in ABCG2 with Co^{2+} ions (32). MRP6 and ABCG2 have been shown to function as active transporters for an anionic cyclic pentapeptide BQ-123 (33) and anticancer drugs (34), respectively. These results suggest that ABCA1 may function as an active transporter in apoA-I-dependent cholesterol release.

W590S ABCA1-GFP showed vanadate-induced nucleotide trapping in the presence of Mn^{2+}. This suggests that the first catalytic reaction to form a stable inhibitory complex ABCA1-MnADP-Vi is not impaired by the mutation. It has been reported that apoA-I does not properly interact with ATP hydrolysis mutants of ABCA1 (10) and that apoA-I can be interacted with ABCA1-W590S as with the wild-type ABCA1 (17, 35). These results suggest that W590S ABCA1-GFP possesses, at least, minimum ATPase activity, which supports apoA-I binding. W590S mutation may impair a step after the interaction with apoA-I, such as proper loading of phospholipid and/or cholesterol or proper release of apoA-I after phospholipid/cholesterol loading.

More than 30 mutations have been mapped in the *ABCA1* gene in patients with FHA and TD (5–7, 13–15). One subgroup of the mutations is suggested to be associated with splenomegaly, and the other may be associated with coronary heart disease (20). Many mutations have been located in ECD1 of ABCA1, and three missense mutations cluster in the vicinity between amino acids 587 and 597 in ECD1. Interestingly, clinical manifestations of these mutations are apparently different (20): R587W is associated with coronary heart disease, whereas W590S is associated with splenomegaly. In this study, we demonstrated that the defect of HDL assembly in R587W and Q597R is due to the impaired localization of ABCA1 to the PM. Subcellular trafficking and vanadate-induced nucleotide trapping in the presence of Mn^{2+} were not impaired in ABCA1-GFP containing the W590S mutation so that W590S seems to have a different type of functional defect. Further characterization of TD mutations for the function of ABCA1 would facilitate understanding of the molecular mechanism for cellular cholesterol release and its homeostasis.

Acknowledgment—We thank Kyowa Hakko Kogyo Co. Ltd. for generating anti-ECD1 polyclonal antibody.

REFERENCES

1. Hara, H., and Yokoyama, S. (1991) *J. Biol. Chem.* **266**, 3080–3086
2. Yokoyama, S. (2000) *Biochim. Biophys. Acta* **1529**, 231–244
3. Francis, G. A., Knopp, R. H., and Oram, J. F. (1995) *J. Clin. Invest.* **96**, 78–87
4. Remaley, A. T., Schumacher, U. K., Stonik, J. A., Farsi, B. D., Nazih, H., and Brewer, H. B., Jr. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 1813–1821
5. Brooks-Wilson, A., Marcil, M., Clee, S., Zhang, L., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J., Molhuizen, H., Loubser, O., Ouelette, B., Fichter, K., Ashbourne-Excoffon, K., Sensen, C., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J., and Hayden, M. (1999) *Nat. Genet.* **22**, 336–345
6. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W., Hahmann, H., Oette, K., Rothe, G., Aslanidis, C., Lackner, K., and Schmitz, G. (1999) *Nat. Genet.* **22**, 347–351
7. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J., Deleuze, J., Brewer, H., Duverger, N., Denefle, P., and Assmann, G. (1999) *Nat. Genet.* **22**, 352–355
8. Oram, J., Lawn, R., Garvin, M., and Wade, D. (2000) *J. Biol. Chem.* **275**, 34508–34511
9. Wang, N., Silver, D., Costet, P., and Tall, A. (2000) *J. Biol. Chem.* **275**, 33053–33058
10. Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001) *J. Biol. Chem.* **276**, 9955–9960
11. Arakawa, R., Abe-Dohmae, S., Asai, M., Ito, J.-I., and Yokoyama, S. (2000) *J. Lipid Res.* **41**, 1952–1962
12. Fielding, P. E., Nagao, K., Hakamata, H., Chimini, G., and Fielding, C. J. (2000) *Biochemistry* **39**, 14113–14120
13. Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) *J. Clin. Invest.* **104**, R25–R31
14. Remaley, A. T., Rust, S., Rosier, M., Knapper, C., Naudin, L., Broccardo, C., Peterson, K. M., Koch, C., Arnould, I., Prades, C., Duverger, N., Funke, H., Assman, G., Dinger, M., Dean, M., Chimni, G., Santamarina-Fojo, S., Fredrickson, D. S., Denefle, P., and Brewer, H. B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12685–12690
15. Brousseau, M. E., Schaefer, E. J., Dupuis, J., Eustace, B., Van Eerdewegh, P., Goldkamp, A. L., Thurston, L. M., FitzGerald, M. G., Yasek-McKenna, D., O'Neill, G., Eberhart, G. P., Weiffenbach, B., Ordovas, J. M., Freeman, M. W., Brown, R. H., Jr., and Gu, J. Z. (2000) *J. Lipid Res.* **41**, 433–441
16. Tanaka, A., Ikeda, Y., Abe-Dohmae, S., Arakawa, R., Sadanami, K., Kidera, A., Nakagawa, S., Nagase, T., Aoki, R., Kioka, N., Amachi, T., Yokoyama, S., and Ueda, K. (2001) *Biochem. Biophys. Res. Commun.* **283**, 1019–1025
17. Fitzgerald, M. L., Morris, A. L., Rhee, J. S., Andersson, L. P., Mendez, A. J., and Freeman, M. W. (2002) *J. Biol. Chem.* **277**, 33178–33187
18. Fitzgerald, M. L., Mendez, A. J., Moore, K. J., Andersson, L. P., Panjeton, H. A., and Freeman, M. W. (2001) *J. Biol. Chem.* **276**, 15137–15145
19. Abe-Dohmae, S., Suzuki, S., Wada, Y., Hiroyuki Aburatani, E. Vance, D., and Yokoyama, S. (2000) *Biochemistry* **39**, 11092–11099
20. Schmitz, G., Kaminski, W. E., and Orso, E. (2000) *Curr. Opin. Lipidol.* **11**, 493–501
21. Taguchi, Y., Yoshida, A., Takada, Y., Komano, T., and Ueda, K. (1997) *FEBS Lett.* **401**, 11–14
22. Takada, Y., Yamada, K., Taguchi, Y., Kino, K., Matsuo, M., Tucker, S. J., Komano, T., Amachi, T., and Ueda, K. (1998) *Biochim. Biophys. Acta* **1373**, 131–136
23. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. (1995) *J. Biol. Chem.* **270**, 19383–19390
24. Hashimoto, K., Uchiumi, T., Konno, T., Ebihara, T., Nakamura, T., Wada, M., Sakisaka, S., Maniwa, F., Amachi, T., Ueda, K., and Kuwano, M. (2002) *Hepatology* **36**, 1236–1245
25. Cai, J., Daoud, R., Alqawi, O., Georges, E., Pelletier, J., and Gros, P. (2002) *Biochemistry* **41**, 8058–8067
26. Hamon, Y., Broccardo, C., Chambenoit, O., Luciani, M., Toti, F., Chaslin, S., Freyssinet, J., Devaux, P., McNeish, J., Marguet, D., and Chimini, G. (2000) *Nat. Cell Biol.* **2**, 399–406
27. Remaley, A. T., Stonik, J. A., Demosky, S. J., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Eggerman, T. L., Patterson, A. P., Duverger, N. J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) *Biochem. Biophys. Res. Commun.* **280**, 818–823
28. Neufeld, E. B., Remaley, A. T., Demosky, S. J., Stonik, J. A., Cooney, A. M., Comly, M., Dwyer, N. K., Zhang, M., Blanchette-Mackie, J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) *J. Biol. Chem.* **276**, 27584–27590
29. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116**, 1071–1080
30. Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995) *Cell* **83**, 129–135
31. Szakacs, G., Langmann, T., Ozvegy, C., Orso, E., Schmitz, G., Varadi, A., and Sarkadi, B. (2001) *Biochem. Biophys. Res. Commun.* **288**, 1258–1264
32. Ozvegy, C., Varadi, A., and Sarkadi, B. (2002) *J. Biol. Chem.*
33. Madon, J., Hagenbuch, B., Landmann, L., Meier, P. J., and Stieger, B. (2000) *Mol. Pharmacol.* **57**, 634–641
34. Litman, T., Druley, T. E., Stein, W. D., and Bates, S. E. (2001) *Cell Mol. Life Sci.* **58**, 931–959
35. Rigot, V., Hamon, Y., Chambenoit, O., Alibert, M., Duverger, N., and Chimini, G. (2002) *J. Lipid Res.* **43**, 2077–2086

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 1, Issue of January 2, pp. 604–611, 2004
Printed in U.S.A.

Human ABCA7 Supports Apolipoprotein-mediated Release of Cellular Cholesterol and Phospholipid to Generate High Density Lipoprotein*

Received for publication, September 5, 2003, and in revised form, October 13, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M309888200

Sumiko Abe-Dohmae‡, Yuika Ikeda§, Michinori Matsuo§, Michi Hayashi‡, Kei-ichiro Okuhira‡, Kazumitsu Ueda§, and Shinji Yokoyama‡¶

From ‡Biochemistry, Molecular Biology and Metabolism 1, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601 and the §Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Apolipoprotein-mediated release of cellular cholesterol and phospholipids was induced in HEK293 cells by expressing human ATP-binding cassette transporter A7 (ABCA7) and ABC transporter A1 (ABCA1) proteins, whether transient or stable, to generate cholesterol-rich high density lipoprotein (HDL). Green fluorescent protein (GFP) attached at their C termini did not influence the lipid release reactions. Transfected ABCA7-GFP induced apolipoprotein-mediated assembly of cholesterol-containing HDL also in L929 cells, which otherwise generate only cholesterol-deficient HDL with their endogenous ABCA1. Time-dependent release of cholesterol and phospholipid by apolipoprotein A (apoA)-I was parallel both with ABCA1 and with ABCA7 when highly expressed in HEK293 cells, but dose-dependent profiles of lipid release on apoA-I and apoA-II were somewhat different between ABCA1 and ABCA7. Analyses of the stable clones with ABCA1-GFP (293/2c) and ABCA7-GFP (293/6c) by using the same vector indicated some differences in regulation of their activities by protein kinase modulators. Dibutyryl cyclic AMP increased ABCA1-GFP and the release of cholesterol and phospholipid in 293/2c but increased neither ABCA7-GFP nor the lipid release in 293/6c. Expression of ABCA1-GFP- and apoA-I-mediated lipid release were enhanced in parallel by phorbol 12-myristate 13-acetate (PMA) in 293/2c cells. In contrast, the same treatment of 293/6c increased ABCA7-GFP, but apoA-I-mediated lipid release was significantly suppressed. Despite these different responses to PMA, all of the effects of PMA were reversed by a specific protein kinase C inhibitor Gö6976, suggesting that the changes were in fact due to protein kinase C activation. A thiol protease inhibitor, *N*-acetyl-Leu-Leu-norleucinal, increased the protein levels of ABCA1-GFP in 293/2c and ABCA7-GFP in 293/6c, indicating their common degradation pathway. The data indicated that human ABCA7 would compensate the function of ABCA1 for release of cell cholesterol in a certain condition(s), but post-transcriptional regulation of their activity is different.

Cholesterol is essential for all animal cells as a membrane constituent for regulation of its general physicochemical properties and functions of specific domains. Cellular cholesterol can be derived by *de novo* synthesis or externally supplied via the uptake of cholesterol-containing lipoprotein particles. In contrast, most of the cells are unable to catabolize cholesterol so that cholesterol molecules must be removed from the cells and transported to the liver for their conversion to bile acids as a major exit route of the body cholesterol. Thus cholesterol transport from the peripheral cells to the liver is an essential part of cholesterol homeostasis, both for cells and for the body. High density lipoprotein (HDL)[1] is believed to play a central role in this pathway.

Lipid-free apolipoproteins with amphiphilic α-helical segments were demonstrated to remove cellular lipids to generate cholesterol-containing HDL (1, 2). This reaction was shown deficient in fibroblasts from patients with familial HDL deficiency, Tangier disease (3, 4), and therefore found essential for generation of plasma HDL. Mutations were identified in one of the members of ATP-binding cassette (ABC) transporter superfamily, ABC transporter A1 (ABCA1), as the cause of Tangier disease and other genetic HDL deficiencies (5–9) so that a role of this protein in the generation of HDL by apolipoprotein-cell interaction became the focus of the HDL research. ABC transporter G1 (ABCG1), an ABC transporter protein of a "half-size" structure family, has also been reported to regulate the apolipoprotein A (apoA)-I-mediated lipid release from lipid-laden macrophages (10). However, it is unclear whether this protein can generate HDL in the absence of ABCA1.

ABC transporter A7 (ABCA7) is another member of the same ABCA subfamily of "full-size" transporter as ABCA1, and its cDNA has been cloned from the human macrophage and spleen cDNA libraries exhibiting high homology to other human ABC transporters (11). ABCA7 has also been identified as the autoantigen SS-N, an epitope of Sjögren's syndrome, which was found homologous to the putative first extracellular domain of ABCA1 (12). ABCA7 mRNA and protein were induced in differentiated macrophages from human peripheral monocytes, and it was apparently expressed inversely to the cellular cholesterol level (11). Although an exact role of ABCA7 in cellular cholesterol homeostasis is unknown, it may play a relevant role

* This work was supported in part by grants-in-aids from the Ministry of Education, Culture, Science and Technology and Ministry of Welfare, Health and Labor of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 81-52-853-8139; Fax: 81-52-841-3480; E-mail: syokoyam@med.nagoya-cu.ac.jp.

[1] The abbreviations used are: HDL, high density lipoprotein; ABC, ATP-binding cassette; ABCA1, ABC transporter A1; ABCG1, ABC transporter G1; ABCA7, ABC transporter A7; GFP, green fluorescent protein; DF, Dulbecco's modified Eagle's medium and Ham's F12 medium; FCS, fetal calf serum; apoA, apolipoprotein A; BSA, bovine serum albumin; lysoPC, lysophosphatidylcholine; dBcAMP, dibutyryl cyclic AMP; PMA, phorbol 12-myristate 13-acetate; ALLN, *N*-acetyl-Leu-Leu-norleucinal; PKC, protein kinase C; PKA, cAMP-dependent protein kinase.

TABLE I

ApoA-I-mediated lipid release from parent HEK293 cells treated with protein kinase stimulants

Cells were subcultured in 6-well trays at a density of 1.0×10^6 cell/well and incubated for 48 h. The cells were washed with buffer H, and 1 ml/well of 0.1 % BSA-DF containing apoA-I (10 μg/mL), dBcAMP (300 μM) and PMA (320 nM) was added as indicated. Medium was collected after 24 h for cholesterol (Ch) and phospholipid (PL) analysis (μg/well). Results shown are the average and variation for two samples.

apoA-I		+	+	+	+
dBcAMP			+		+
PMA				+	+
Ch	0.02 ± 0.01	−0.01 ± 0.03	0.05 ± 0.03	0.02 ± 0.00	0.02 ± 0.01
PL	0.53 ± 0.00	0.62 ± 0.12	0.69 ± 0.07	0.62 ± 0.07	0.59 ± 0.03

in regulation of cholesterol turnover in some specific cells such as macrophages.

Recently, it was reported that mouse ABCA7 promotes apoA-I-mediated phospholipid release but failed to release cholesterol (13). Here we report that human ABCA7 mediates apolipoprotein-dependent generation of HDL by releasing both cellular cholesterol and phospholipid even in the absence of ABCA1. Although the reaction is largely similar to that of human ABCA1, apparent affinity of apolipoproteins for the cells is different. Regulations of its expression and activity by protein kinase activators are also different from those of ABCA1.

EXPERIMENTAL PROCEDURES

DNA Construction and Transfection—Full-length cDNAs for human ABCA1 and ABCA7 were cloned as described previously (12). They were introduced to pcDNA3.1/Hygro (Invitrogen), pCMV6c, and pEGFP-N1 (Clontech) to obtain constructs for the proteins without or with green fluorescent protein (GFP) at their C terminus. All the vectors have an immediate early promoter of cytomegalovirus promoter for expression of cDNA. HEK293 and L929 cells were obtained from Health Science Research Resources Bank and maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) supplemented with 10% (v/v) of fetal calf serum (FCS, Invitrogen) under a humidified atmosphere of 5% CO_2, 95% air at 37 °C. cDNAs were transfected with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instruction. Cells to which the cDNAs were permanently introduced were selected with G418, and clones with high level expression of the fusion proteins were further selected according to the fluorescent intensity with GFP.

Detection of Expressed Proteins—Western blotting was carried out with an anti-ABCA1 antiserum (14, 15), a rat polyclonal anti-ABCA7 antibody, and an anti-GFP antibody (Santa Cruz Biotechnology) to indicate the expression levels of the proteins. To generate the anti-ABCA7 antibody, the putative first extracellular domain of human ABCA7, amino acids 45-459, was expressed as a fusion protein with His tag at the C terminus in *Escherichia coli*, purified by Ni^+ chromatography (Qiagen),[2] and used for immunization. Expression levels of ABCA1-GFP protein and ABCA7-GFP protein were also measured *in situ* with an FL600 fluorescent plate reader (Bio-Tek Inc.). Cellular fluorescence was measured as fluorescent intensity per unit area. GFP-derived derived fluorescence was calculated by subtracting background. Intracellular localization of GFP-containing protein was examined by their fluorescence images obtained by using an Axiovert microscope (Carl Zeiss) equipped with a MicroRadiance confocal laser scanning microscope (Bio-Rad).

Apolipoproteins—ApoA-I and apoA-II were isolated from human plasma HDL fraction (density 1.09–1.21) and stored at −80 °C until use as described previously (16, 17). Stock solutions (1 mg/ml) were prepared and stored at 4 °C as described previously (18).

Cellular Lipid Release Assay—Cells were subcultured in 6-well trays (TPP catalogue number 92406) at a density of 1.0×10^6 cells/well with 10% FCS-DF medium. After a 48-h incubation, the cells were washed once with buffer H (Hank's balanced salt solution containing 20 mM HEPES-KOH (pH 7.5) and 14 mM glucose), and incubated in 1 ml/well of DF medium containing 0.02% bovine serum albumin (BSA) and lipid acceptors (apoA-I, apoA-II, and 2-hydroxypropyl-β-cyclodextrin). Buffer H supplemented with amino acids (Invitrogen catalogue number 11140-50) was used instead of DF medium in some experiments to reduce the endogenous fluorescence background in DF medium. Lipid content in the medium and cells was determined after the indicated incubation times. Procedures for lipid extraction and enzymatic assays for cholesterol and choline-phospholipids were described previously (18). Enzyme assay in combination with lysophospholipase, glycerophosphorylcholine phosphodiesterase, and choline oxidase (19) was also applied to evaluate the level of lysophosphatidylcholine (lysoPC), a causal molecule for background in choline-phospholipid assay.

Density Gradient Analysis—Cells were subcultured in 100-mm dishes (TPP catalogue number 93100) at a density of 6.0×10^6 cells/dish, cultured as above and stimulated with 5 ml/dish of DF medium containing 0.1% BSA and 10 μg/ml apoA-I for 24 h. Medium from two dishes was combined and centrifuged to remove cell debris, and 8 ml of the supernatant was processed for sucrose density gradient ultracentrifuge (1). The solution was collected from the bottom into 13 fractions. The contents of cholesterol and choline-phospholipids as well as the density were determined for each fraction.

Statistical Analysis—Data were analyzed by one-way analysis of variance followed by Scheffé's test. A p value less than 0.05 was accepted as statistically significant.

RESULTS

Parent HEK293 cells did not respond to apolipoproteins to release either cholesterol or phospholipid (Table I). Treatment with dibutyryl cyclic AMP (dBcAMP) and phorbol 12-myristate 13-acetate (PMA) with or prior to apoA-I stimulation had no effect either (Table I and data not shown). Transient expression of ABCA1 cDNA and ABCA7 cDNA in HEK293 cells resulted in apoA-I-mediated release of both cholesterol and phospholipid in a dose-dependent manner (Fig. 1), and attachment of GFP to the C terminus of ABCA7 or ABCA1 did not influence these lipid releases (see below). For further investigation of the functions of ABCA7, we therefore obtained stable clones expressing high levels of ABCA1-GFP protein and ABCA7-GFP protein.

Release of cholesterol and phospholipid by incubation with apoA-I was demonstrated at least in three independent clones highly expressing ABCA1-GFP and also three clones expressing ABCA7-GFP, indicating that the reaction is not clone-specific but cDNA-specific. Western blotting data of parent HEK293 cells, the cells with or without transient expression of ABCA1 and ABCA7, and ABCA1-GFP-expressing clone (293/2c) and ABCA7-GFP-expressing clone (293/6c) are shown in Fig. 2. Neither ABCA1 nor ABCA7 protein was detected in parent HEK293 cells. ABCA1- and ABCA7-containing bands were detected at the position consistent with those of GFP-attached molecules in 293/2c and 293/6c cells, as ABCA1-GFP and ABCA7-GFP at 260–270 and 240–260 kDa, respectively (Fig. 2, *C–E*). ABCA1 protein expression level per cell protein in 293/2c cells was estimated as about 30-fold of that in human fibroblast WI38 and mouse fibroblast L929 by Western blotting analysis (data not shown). GFP protein expression was ~1:3 between 293/2c and 293/6c cells based on Western blotting analysis results (Fig. 2*C*) and measurement of fluorescence (see Figs. 7*C* and 9*C*, compare the group 0). Confocal microscopic analysis revealed that ABCA1-GFP (20) and ABCA7-GFP (Fig. 2*F*) were localized mainly in plasma membrane, as was recently reported in CHO cells stably expressing rat ABCA7 (21) and in HEK293 cells transiently expressing mouse ABCA7 (13).

Typical profiles of lipid release by apoA-I and apoA-II from 293/2c and 293/6c cells are shown in Fig. 3. LysoPC assay

[2] R. Aoki and K. Ueda, unpublished results.

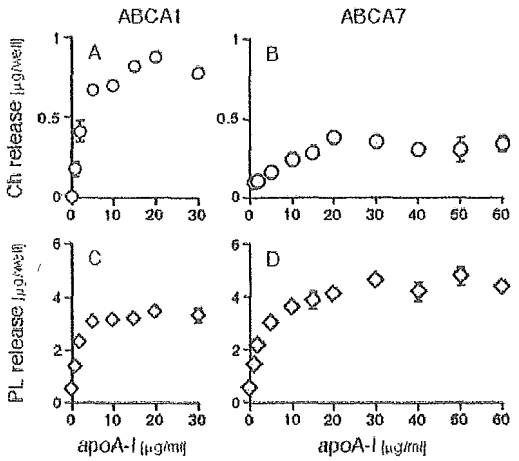

FIG. 1. **ApoA-I-mediated release of cholesterol (*Ch*, *A* and *B*) and choline-phospholipids (*PL*, *C* and *D*) from HEK293 cells transiently expressing ABCA1 cDNA (*A* and *C*) and ABCA7 cDNA (*B* and *D*).** Parent HEK293 cells were subcultured in 6-well trays at a density of 1.0×10^6 cell/well and incubated for 24 h. The cells were transfected with 1.0 μg/well of ABCA1/pcDNA3.1/Hygro (*A* and *C*) and ABCA7/pCMV6c (*B* and *D*). After a 24-h incubation, the cells were washed with buffer H (see "Experimental Procedures"), 1 ml/well of 0.02% BSA-DF containing various concentrations of apoA-I as indicated. Medium was collected after 24 h for lipid analysis. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I-incubation (48 h after subculture) were 12.0 ± 0.5 and 92.3 ± 3.3 μg/well in ABCA1/pcDNA3.1/Hygro-transfected cells, and 12.8 ± 0.4 and 105.5 ± 1.4 μg/well in ABCA7/pCMV6c-transfected cells, respectively (average ± S.D. for six samples).

confirmed that most of the choline-phospholipids released to the medium in the absence of apolipoproteins was lysoPC and that its level was independent of apolipoprotein concentration (data not shown). The dose-dependent curve of the reaction by apoA-II was similar to that of apoA-I with respect to molar concentration of the proteins for both cholesterol and choline-phospholipid (Fig. 3). These results indicated that ABCA7 directly promotes both cholesterol and phospholipid efflux to apoA-I, just as ABCA1 does. The releases of phospholipid and cholesterol appear parallel by increasing concentrations of apoA-I and apoA-II in 293/2c (ABCA1-GFP) and 293/6c (ABCA7-GFP). The lipid release seems to reach the maximum at lower concentration of apolipoprotein with the ABCA1-expresing cells (293/2c) than the ABCA7-expressing cells (293/6c). The EC_{50} for the apoa-I mediated release of cholesterol and phospholipid from 293/6c cells was ~4.5 and 4.2 μg/ml (0.16 and 0.15 μM), respectively, whereas 1.8 and 1.2 μg/ml (0.064 and 0.043 μM) for that from 293/2c cells (Fig. 3 and data not shown). Time course analysis of apoA-I-mediated lipid release supported the idea of simultaneous release of phospholipid and cholesterol both from 293/2c cells and from 293/6c cells as cholesterol and phospholipid in the medium were well detectable as early as 1 h after apoA-I stimulation, and they increased linearly for 8 h (Fig. 4). Density gradient analysis of the medium demonstrated that both cholesterol and phospholipid were recovered in the fractions with a density peak at around 1.08 g/ml, indicating that HDL particles were generated from the ABCA7-GFP-expressing cells similarly to the ABCA1-GFP-expressing cells (Fig. 5).

Expression of ABCA7-GFP also induced cholesterol release in L929 cells. We reported elsewhere that L929 cells release phospholipid but not cholesterol by apoA-I (22). As shown in Fig. 6, parent L929 cells released only phospholipid to apoA-I even at high concentrations of apoA-I (*open symbols*). Stable expression of ABCA7-GFP protein in L929 cells caused substantial release of cholesterol together with enhancement of phospholipid release (*closed symbols*). The level of endogenous ABCA1 expression was not affected in L929 by transfection and expression of ABCA7 (Fig. 6*C*).

Both dBcAMP and PMA enhanced apoA-I-mediated lipid release and ABCA1-GFP protein level in 293/2c. In the presence of dBcAMP (300 μM) or PMA (320 nM) with apoA-I, significant increase was induced in cholesterol and phospholipid release from 293/2c cells (Fig. 7, *A* and *B*, *open columns*). The increase of the lipid release was correlated with elevation of ABCA1-GFP protein level evaluated by GFP-derived fluorescence intensity and Western blotting analysis (Figs. 7*C* and 8*A*, *lanes 2–7*). The effects of dBcAMP and PMA were slightly synergistic, although not additive, in the condition tested.

Previous works demonstrated protection of ABCA1 by apolipoprotein against thiol protease-mediated degradation (15, 22–24). ApoA-I increased protein levels of ABCA1 (Fig. 8*A*) and ABCA1-GFP (Fig. 7*C* and other data not shown) expressed in HEK293 cells, consistent with the previous similar experiments (23, 24). A thiol protease inhibitor, *N*-acetyl-Leu-Leu-norleucinal (ALLN), enhanced apoA-I-mediated lipid release and ABCA1-GFP protein level as well as the increase of GFP-derived fluorescence in 293/2c (Figs. 7, *A–C*, and 8*A*).

In contrast, treatment with dBcAMP did not affect apoA-I-mediated cholesterol or phospholipid release in 293/6c cells, and PMA decreased release of both lipids (Fig. 9, *A* and *B*, *shadowed columns*). On the other hand, apoA-I and ALLN caused the increase of ABCA7-GFP in 293/6c, although the increase in cholesterol and phospholipid release was not statistically significant (Figs. 8*B*, 9, *A* and *B*, *hatched columns*). ABCA7-GFP protein level was not significantly affected either by dBcAMP, whereas it was slightly up-regulated by PMA (Figs. 8*B* and 9*C*). None of the compounds tested influenced non-specific cholesterol release to 2-hydroxypropyl-β-cyclodextrin from 293/2c or 293/6c (data not shown). These effects of dBcAMP, PMA, and apoA-I were similar in HEK293 cells transiently or stably expressing ABCA7 (Fig. 8*B*, *lanes 1–4*, and data not shown)

Induction of ABCA1 and ABCA7 by dBcAMP or PMA was further investigated in 293/2c and 293/6c cells, respectively, by monitoring their GFP-derived fluorescence (Fig. 10). In 293/2c cells, PMA induced the transient increase of ABCA1-GFP fluorescence, as the increase was evident after 2 h, reaching a peak at 8–12 h (Fig. 10*A*). dBcAMP-induced fluorescence increase was rather continuous at least up to 30 h after a time lag of 4–6 h. The effect of these two compounds were additive. Continuous monitoring of cellular fluorescence in the fluorescence-free medium yielded similar results (data not shown). Being consistent with these results, the apoA-I-mediated lipid release from 293/2c for 4 h was not affected by dBcAMP but was enhanced by PMA (data not shown). In 293/6c cells, fluorescence level was changed neither by dBcAMP nor by PMA (Fig. 10*B*). Fluorescent levels were increased in parallel with lipid release in 293/2c cells when treated with ALLN, whereas the increase was slight and unsustainable in 293/6c (data not shown).

Gö6976, a PKC inhibitor (25), reversed all of the changes caused by PMA. In 293/2c cells, the enhancement of apoA-I-mediated lipid release and ABCA1-GFP induction was inhibited (Figs. 8*A* and 11, *A–C*). In 293/6c cells, suppression of the apoA-I-mediated lipid release by PMA was recovered (Fig. 11, *D* and *E*), whereas the ABCA7-GFP level was reduced to the control level (Figs. 8*B* and 11*F*). Gö6976 alone had no signifi-

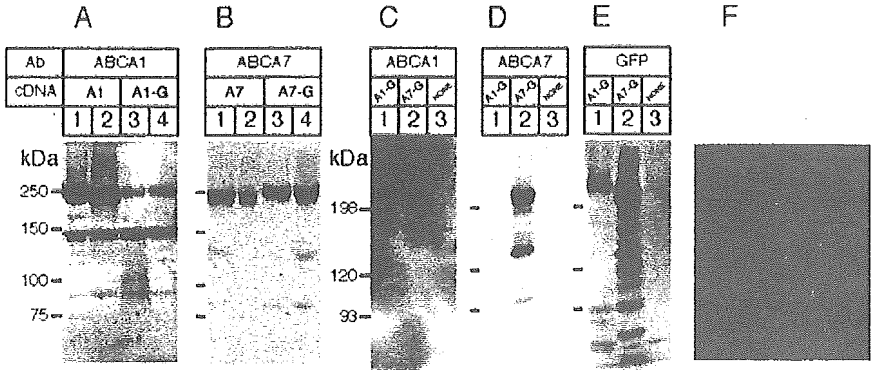

FIG. 2. **Detection of ABCA1- and ABCA7-containing proteins in HEK293 cells.** *A* and *B*, Western blotting analysis of transiently expressed proteins. Parent HEK293 cells were cultured and transfected with ABCA1/pcDNA3.1/Hygro (*A*, *lanes 1* and *2*), ABCA1-GFP/pcDNA3.1 (*A*, *lanes 3* and *4*), ABCA7/pCMV6c (*B*, *lanes 1* and *2*), and ABCA7-GFP/pcDNA3.1 (*B*, *lanes 3* and *4*) as in Fig. 1. Cells were collected after 30 h for crude membrane preparation. Membrane proteins (80 μg for *A*, *lanes 1* and *2* and *B*; 40 μg for *A*, *lanes 3* and *4*) were separated on a 5.5% SDS-polyacrylamide gel, transferred onto Immobilon (Millpore), and analyzed by using rabbit anti-ABCA1 antiserum (*A*) and rat polyclonal anti-ABCA7 antibody (*Ab*) (*B*). *C–E*, Western blotting analysis of 293/2c, 203/6c, and parent HEK293 cells. Cells were cultured in 10% FCS-DF and processed to prepare crude membrane fraction. 20 μg of membrane proteins were separated as above and analyzed by using rabbit anti-ABCA1 antiserum (*C*), rat polyclonal anti-ABCA7 antibody (*D*), and mouse monoclonal anti-GFP antibody (*E*). *Lane 1*, 293/2c; *lane 2*, 293/6c; *lane 3*, parent HEK293. *F*, subcellular localization of ABCA7-GFP protein. The 293/6c cells were cultured on a 35-mm glass-dish (Iwaki). Fluorescence images of the GFP were obtained as described under "Experimental Procedures."

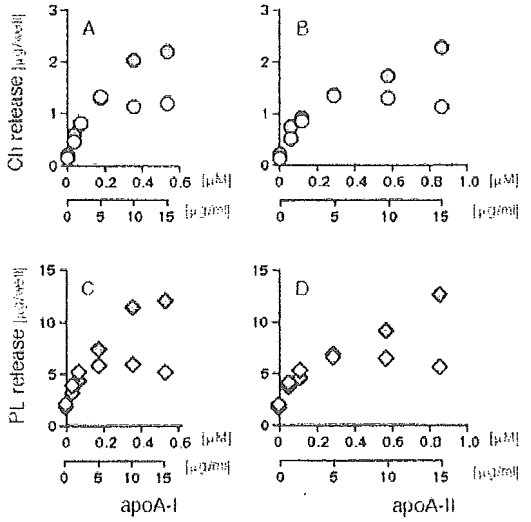

FIG. 3. **Release of cholesterol (*Ch*, *A* and *B*) and choline-phospholipids (*PL*, *C* and *D*) from HEK293 cells mediated by apoA-I (*A* and *C*) and apoA-II (*B* and *D*).** Cells were subcultured in 6-well trays at a density of 1.0×10^5 cell/well and incubated for 48 h. After washing with buffer H, cells were incubated in 1 ml/well of 0.02% BSA-DF containing apolipoproteins at the concentrations indicated. Concentrations of cholesterol and choline-phospholipid in the medium from 293/2c (*open symbols*) and 293/6c (*shadowed symbols*) cells were determined after 16 h. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I incubation were 9.2 ± 0.6 and 40.4 ± 2.4 μg/well in 293/2c cells and 9.2 ± 0.5 and 36.2 ± 2.3 μg/well in 293/6c cells, respectively (average ± S.D. for eight samples).

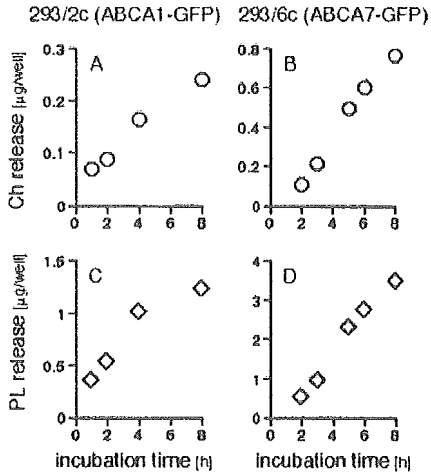

FIG. 4. **Simultaneous and time-dependent increase in apoA-I-mediated release of cholesterol (*Ch*) and choline-phospholipid (*PL*) from 293/2c and 293/6c cells.** 293/2c (*A* and *B*) and 293/6c (*C* and *D*) cells were cultured for 48 h as in Fig. 3. After washing with buffer H, cells were incubated in 0.02% BSA-DF containing 10 μg/ml apoA-I. After the incubation time indicated, cholesterol (*A* and *C*) and choline-phospholipid (*B* and *D*) content in the medium was measured. Results shown are representative from two independent sets of experiments after subtraction of the background. Each data point is from a single sample for 293/2c and from two samples for 293/6c.

cant effects on apoA-I-mediated lipid release from 293/2c or 293/6c (Fig. 11).

DISCUSSION

The function and its regulation of human ABCA7 was studied by using its expressing system of in HEK293 cells. ABCA7 exhibited a function for generation of cholesterol-containing HDL upon the interaction with apoA-I and apoA-II so much as human ABCA1 does. Response of ABCA7 to protein kinase modulators, dBcAMP and PMA, was somewhat different from ABCA1. ABCA1 protein level was increased by either reagent, and its function for mediating the apoA-I-mediated lipid release was increased in parallel, whereas ABCA7 and its activity were not increased by dBcAMP. Interestingly, ABCA7 was slightly increased by PMA, but its activity for mediating lipid release by apolipoprotein was rather suppressed. All of the effects of PMA were reversed by PKC-specific inhibitor Gö6976, indicating that they are mediated by PKC.

Deficiency of ABCA1 causes loss of plasma HDL, as demonstrated in the patients with Tangier disease (5–9) and in ABCA1 knockout mice (26, 27), to indicate that there is no significant compensatory backup system for supply of plasma HDL. At the cellular level, however, ABCG1 may function as a

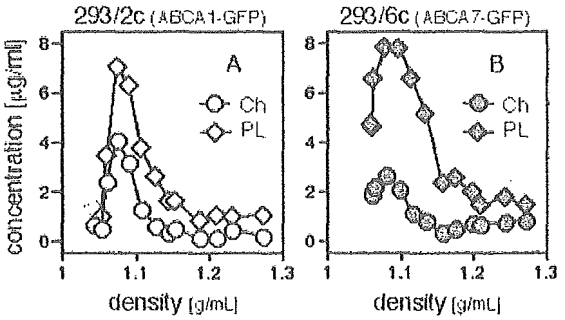

FIG. 5. **Density gradient analysis of lipids released from 293/2c (*A*) and 293/6c (*B*) cells.** Cells were cultured and stimulated with 10 μg/ml apoA-I. Medium was processed as described under "Experimental Procedures."

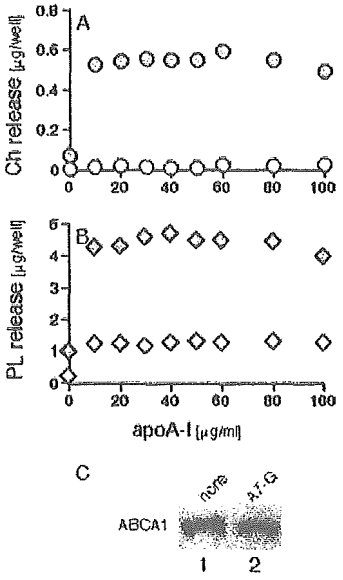

FIG. 6. **Effect of ABCA7 expression in L929 cells.** *A* and *B*, release of cholesterol (*Ch*, *A*) and choline-phospholipids (*PL*, *B*) mediated by apoA-I from parent L929 cells (*open symbols*) and L929 cells stably expressing ABCA7-GFP protein (mixture of several clones)(*shadowed symbols*). Cells were subcultured in 6-well trays at a density of 5.0×10^5 cell/well and processed as in Fig. 3. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I-incubation were 12.1 ± 0.5 and 43.3 ± 0.4 μg/well in parent L929 cells and 10.3 ± 0.2 and 68.3 ± 3.7 μg/well in L929 cells stably expressing ABCA7-GFP, respectively (average ± S.D. for six samples). *C*, ABCA1 protein level in L929 cells. Parent L929 cells (*lane 1*) and ABCA7-GFP-expressing L929 cells (*lane 9*) were cultured and processed as described in the legend for Fig. 2. Membrane protein (100 μg) was analyzed by Western blotting for each cell line cells.

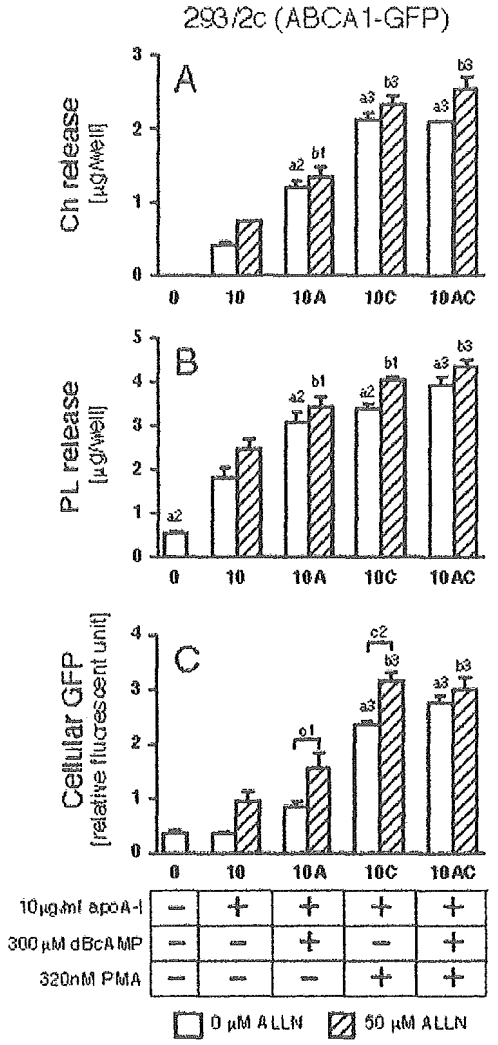

FIG. 7. **Effects of dBcAMP, PMA and ALLN on apoA-I-mediated lipid release and ABCA1-GFP protein level in 293/2c cells.** 293/2c cells were cultured for 48 h as described for Fig. 3. The cells were then washed with buffer H, cultured in 0.02% BSA-DF without (*open columns*) or with (*hatched columns*) 50 μM ALLN and reagents indicated. Medium was collected after a 9-h incubation for lipid analysis. Remaining cells were washed with buffer H, and GFP-derived fluorescence was measured. Results represent the average and variation for two samples of the lipid release and means ± S.D. for three samples of cellular GFP, respectively. *a1*, *a2*, and *a3*, $p < 0.05$, 0.01, and 0.001 *versus* group 10 without ALLN; *b1*, *b2*, and *b3*, $p < 0.05$, 0.01, and 0.001 *versus* group 10 with ALLN; *c1*, *c2*, *c3*, $p < 0.05$, 0.01, and 0.001 among the groups indicated. *Ch*, cholesterol; *PL*, phospholipid.

regulator of lipid transport in lipid-laden macrophages, although its *in vivo* function is not defined. Suppression of ABCG1 expression with the ABCG1-specific antisense oligonucleotide caused 32 and 25% reduction of the release of cholesterol and phospholipid, respectively, whereas ABCA1 expression was not down-regulated (10). It was also shown that levels of ABCG1 mRNA in non-cholesterol-laden macrophages from two patients with Tangier disease were significantly greater than controls, although the function of ABCG1 was not examined (28).

We demonstrated that human ABCA7 mediates the apolipoprotein-dependent cellular lipid release and consequent assembly of new HDL in a very similar manner as human ABCA1 *in vitro*. Our experimental protocol fulfills the condition to observe the effect of ABCA7 in the absence of ABCA1 so that this is an isolated function of this protein, at least *in vitro*. The results from L929 cells expressing ABCA7-GFP in addition to their endogenous ABCA1 (Fig. 6) suggested that the effects of ABCA1 and ABCA7 in apolipoprotein-mediated HDL generation may be synergistic. However, it is not clear whether ABCA1 and ABCA7 generate HDL particles independently, cholesterol-deficient HDLs and cholesterol-containing HDLs in this case. The reaction mechanisms of ABCA1 and ABC7 should be further investigated, including their difference, cross-talk, and physiological relevance.

In HEK293 cells, release of lipid may reach maximum at a

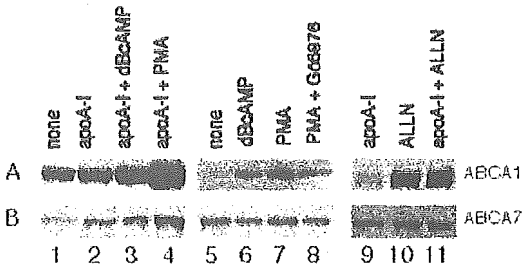

FIG. 8. **Effects of apoA-I, dBcAMP, PMA, Gö6976, and ALLN on ABCA1 and ABCA7 protein levels.** HEK293 cells stably expressing ABCA1 (mixture of several clones, *A, lanes 1–4*), ABCA1-GFP (clone 293/2c, *A, lanes 5–11*), ABCA7 (mixture of several clones, *B, lanes 1–4*), and ABCA7-GFP (clone 293/6c, *B, lanes 5–11*) were cultured as described under "Experimental Procedures." After a 48-h incubation, cells were washed with buffer H, and then 0.02% BSA-DF medium containing the compounds indicated was added. The final concentration of apoA-I, dBcAMP, PMA, Gö6976 and ALLN was 10 μg/ml, 300 μM, 320 nM, 10 μM, and 50 μM, respectively. Cells were cultured for another 16 h and collected for membrane preparation. ABCA1 (*A*) and ABCA7 (*B*) protein were detected by Western blotting.

slightly lower concentration of apoA-I and apoA-II (2–5 μg/ml) when ABCA1 was expressed than in the cells expressing ABCA7 (maximum at 20–30 μg/ml apoA-I) (Figs. 1 and 3). The results with ABCA1 were apparently consistent with previous findings with RAW264 where ABCA1 was strongly induced by dBcAMP, as shown by oligonucleotide array analysis that the mRNA level was increased 10-fold, whereas ABCA7 mRNA level was low and not affected (18). A similar dose-dependent curve was observed with human fibroblast WI-38 cells in which ABCA1 was found (22) but not ABCA7 (data not shown). Further investigation is required for understanding the underlying mechanism for this apparent difference in kinetic profiles of the HDL assembly reactions between ABCA1 and ABCA7.

HEK293 cells transiently expressing mouse ABCA7 released phospholipids but not cholesterol by apoA-I, even in the presence of scavenger receptor-BI or loading of extra cholesterol mass (13). As HEK293 cells transiently expressing mouse ABCA1 were able to generate cholesterol-containing HDL (13, 29), the difference may be between human ABCA7 and mouse ABCA7.

The effect of dBcAMP on ABCA1-GFP level in 293/2c is consistent with the previous reports, an increase of ABCA1 mRNA in human fibroblasts (22), RAW264 cells (18, 30), and macrophages (31) by cAMP analogues. It has been reported that there is a cAMP-responsive element in the ABCA1 promoter (32). However, neither ABCA1 protein (Fig. 1 and data not shown) nor apoA-I-mediated HDL generation (Table I) was detected in parent HEK293 cells even after the dBcAMP treatment so that it is unlikely that the enhancement of the ABCA1 activity by dBcAMP in 293/2c is carried out by this cAMP-responsive element of the endogenous ABCA1 gene. In fact, no immunoreactive band was detected in 293/2c at the position of lower molecular weight than ABCA1-GFP even after the cAMP treatment. The promoters of the transfected cDNAs were common (immediate early promoter of cytomegalovirus) for ABCA1-GFP and ABCA7-GFP and dBcAMP did not increase ABCA7-GFP so that the effect of cAMP in this case is likely to be on the post-transcriptional modulation.

ABCA1 protein expressed in *Xenopus* oocytes was phosphorylated by PKA in a cell-free system (33), and its activity as an anion transporter was enhanced after short term treatment with PKA activators (33). Also, 8-bromo-cAMP promoted phosphorylation of ABCA1 by a 1-h incubation in normal human fibroblasts and increased the apoA-I-mediated release of cholesterol and phospholipid without changing its mRNA or protein level (34). A more recent report indicated that phosphorylation by PKA at a specific site of ABCA1 is constitutive but important for the apoA-I-mediated phospholipid release (35). Our results seem rather consistent with the cellular conditions similar to *Xenopus* oocytes (33) or fibroblasts (34). Regulation of ABCA7 by PKA may then be different in this regard.

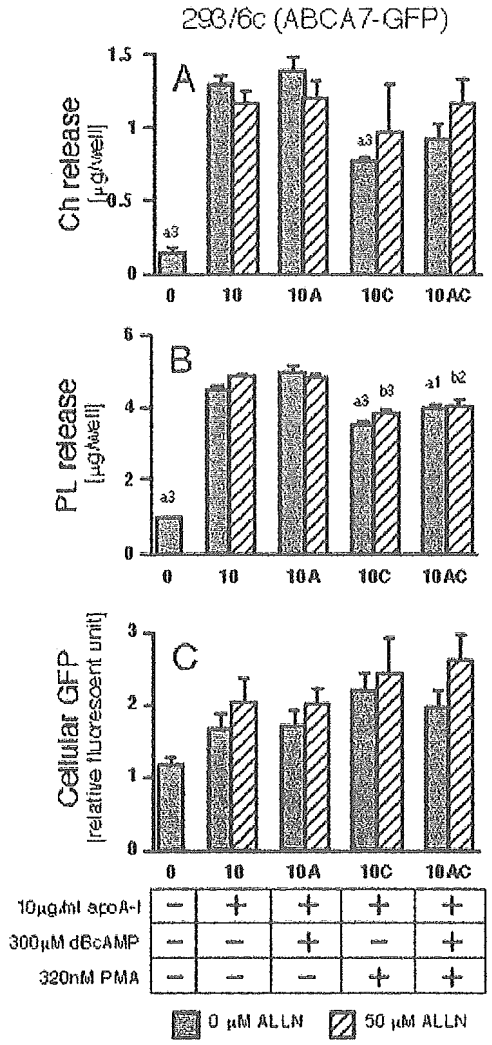

FIG. 9. **Effects of dBcAMP, PMA and ALLN on apoA-I-mediated lipid release and ABCA7-GFP protein level in 293/6c cells.** 293/6c cells were processed in the same way as in Fig. 7. Results represent the average and variation for two samples. Legends are same as in Fig. 7. *Ch*, cholesterol; *PL*, phospholipid.

The effect of the PKC activator, PMA, also differentiated the response of ABCA1 and ABCA7. ABCA1-GFP in 293/2c was increased by PMA, and the activity also seemed increased in parallel (Fig. 7). In contrast, PMA decreased the apoA-I-mediated lipid release from 293/6c expressing ABCA7-GFP (Fig. 9, *A* and *B*), whereas it did not cause significant reduction of the GFP-derived fluorescence (Figs. 9*C* and 11*F*). These effects of PMA were all reversed by Gö6976, an inhibitor of Ca^{2+}-dependent isoform(s) of PKC (25). The data indicate that specific activities of ABCA7 can be modulated by PKC.

Turnover of ABC transporters has not been fully understood. We recently reported that degradation of ABCA1 is protected by apolipoproteins from the degradation by thiol protease, most likely calpain (15, 24). ALLN was effective to increase ABCA1

protein expressed in HEK293 cells, indicating that ABCA1 protein expressed by the exogenously transfected cDNA is metabolized by the similar mechanism, being consistent with other reports (23, 24). The results with the transfected ABCA7 were largely similar to those with ABCA1, showing that the metabolic pathway of these proteins are common.

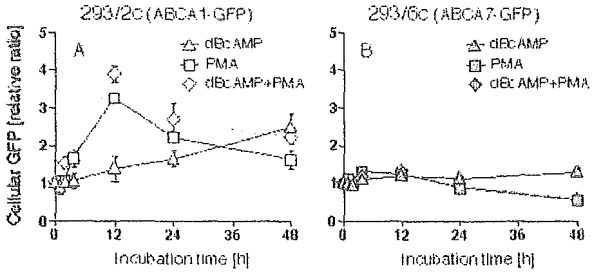

FIG. 10. **Time course analysis in GFP-derived fluorescence in 293/2c (*A*) and 293/6c (*B*).** The clone cells were subcultured into 12-well trays 5.0×10^5 cells/well and cultured for 64 h in 10% FCS-DF. Reagents indicated were added into the wells at the time 16, 40, 52, 60, 62, and 63 h after subculture. The final concentrations of dBcAMP and PMA were 300 μM and 320 nM, respectively. At 64 h of the incubation after subculture, medium was removed, cells were washed with buffer H, and cellular fluorescence was measured. Data are relative to control cells that were maintained in 10% FCS-DF throughout the experiment. In this experimental condition, a 48-h incubation with dBcAMP did not affect cell growth, whereas PMA suppressed it by about 15%. These effects were same for parent HEK293 cells, 293/2c cells, and 293/6c cells. Results represent means ± S.D. for three samples.

The physiological relevance of the activity of ABCA7 reported in this study should be further investigated. ABCA1 distribution is ubiquitous (33), and its dysfunction results in the loss of plasma HDL (5–9), whereas tissue distribution of ABCA7 is reportedly restricted to myelo-lymphatic tissues in human (11) and mouse (36) or preferentially in platelets in rat (21) and mouse (13). Therefore, the ABCA7-mediated lipid release may not contribute significantly to a source of plasma HDL. However, it may still play an important role in cellular cholesterol homeostasis in particular tissues including macrophages. In Tangier disease, accumulation of cholesteryl ester is found in foamy histiocytes in the reiticuloendotherial system, fibroblasts of the cornea, melanocytes, Schwann cells, neurons, and non-vascular-muscle cells (37). It is interesting to point out that Tangier disease patients appear to be only at moderately increased cardiovascular risk despite the almost complete loss of plasma HDL and considerable cholesterol ester accumulation in resident macrophages of many tissues (37). ABCA1 knockout mice showed tissue distribution of lipid deposition identical to Tangier disease and no abnormalities in aorta even in aged mice (38). These findings may suggest that there is a protecting system against the development of atherosclerosis even in the absence of ABCA1, and the ABCA7-mediated lipid

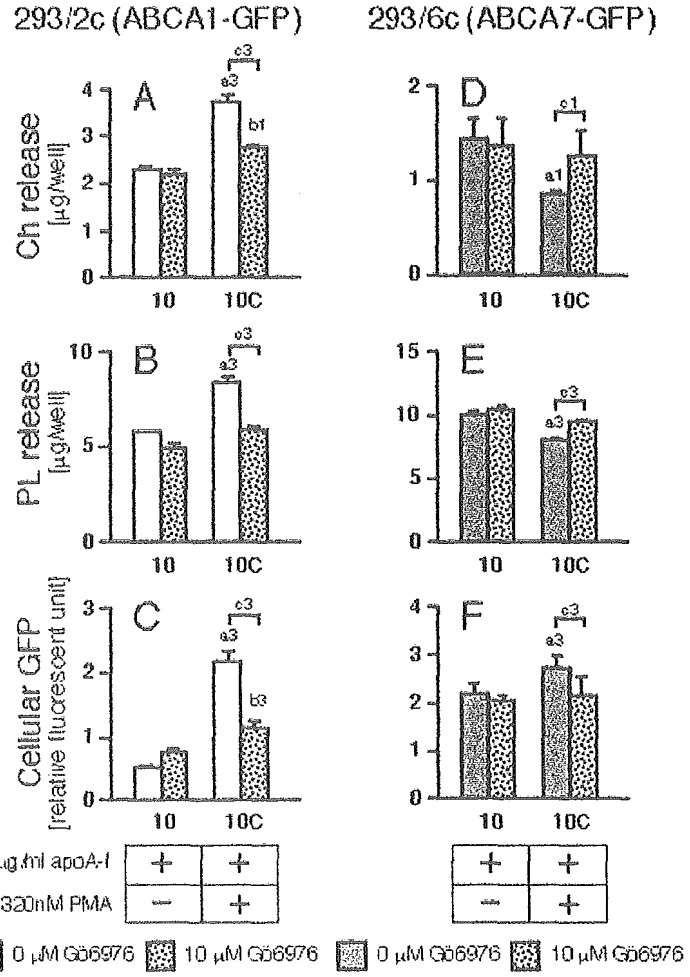

FIG. 11. **Effects of Gö6976 on the apoA-I-mediated lipid release and the GFP protein level for 293/2c (*A–C*) and 293/6c (*D–F*).** The cells were cultured for 48 h as described in the legend for Fig. 3. The cells were then washed with buffer H, cultured in 0.02% BSA-DF without (*open columns*) or with (*hatched columns*) 10 μM Gö6976 and substances indicated. Medium was collected after an 18-h incubation for lipid analysis. Remaining cells were processed as in Fig. 7. Results represent means ± S.D. for three and six samples in lipid release and cellular GFP, respectively. *a1* and *a3*, $p < 0.05$ and 0.001 *versus* group 10 without Gö6976; *b1* and *b3*, $p < 0.05$ and 0.001 *versus* group 10 with Gö6976; *c1* and *c3*, $p < 0.05$ and 0.001 between the groups indicated. *Ch*, cholesterol; *PL*, phospholipid.

release from macrophages in the vascular wall is one of the candidates. In fact, ABCA7 protein is detected in peripheral blood monocytes after *in vitro* differentiation into macrophages followed by acetylated LDL loading (11). Specific roles of this protein should be examined, especially in atherosclerotic tissues.

Further study of the mechanism by which ABCA7 mediates the release of cholesterol and generation of HDL should provide us with important information for intracellular trafficking and homeostasis of cholesterol by comparing it with ABCA1. It would also lead us to novel strategies for treatment of atherosclerosis. Controlled induction of ABCA7 in certain specific organs, tissues, or cells, such as macrophages and lymphomyeloid cells, would be efficient to remove cholesterol from peripheral tissues to prevent atherosclerosis, by itself or in coordination with ABCA1.

Acknowledgments—We thank Kyowa Hakko Co. Ltd. for generating anti-ABCA7 polyclonal antibody and Tatsuya Kishimoto at ASWELL Inc. for providing reagents for lysoPC assay. We are also grateful to Michiyo Asai for preparation of apolipoproteins. Naotaka Ninagawa, Kaoru Kuzuya, Hiroyuki Imafuji, and Akemi Miyata, students of Nagoya City University Medical School, contributed to the initial stage of the project.

REFERENCES

1. Hara, H., and Yokoyama, S. (1991) *J. Biol. Chem.* **266,** 3080–3086
2. Yokoyama, S. (2000) *Biochim. Biophys. Acta* **1529,** 231–244
3. Francis, G. A., Knopp, R. H., and Oram, J. F. (1995) *J. Clin. Invest.* **96,** 78–87
4. Remaley, A. T., Schumacher, U. K., Stonik, J. A., Farsi, B. D., Nazih, H. B., and Brewer, H. B. J. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 1813–1821
5. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Böttcher, A., Diederich, W., Drobnik, W., Barlage, S., Büchler, C., Porsch-Özcürümez, M., Kaminski, W. E., Hahmann, H. W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K. J., and Schmitz, G. (1999) *Nat. Genet.* **22,** 347–351
6. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L.-H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O. F., Loubser, O., Ouelette, B. F. F., Fichter, K., Ashbourne-Excoffon, K. J. D., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J. P., Genest, J., Jr., and Hayden, M. R. (1999) *Nat. Genet.* **22,** 336–345
7. Marcil, M., Brooks-Wilson, A., Clee, S. M., Roomp, K., Zhang, L. H., Yu, L., Collins, J. A., van Dam, M., Molhuizen, H. O., Loubster, O., Ouellette, B. F., Sensen, C. W., Fichter, K., Mott, S., Denis, M., Boucher, B., Pimstone, S., Genest, J., Jr., Kastelein, J. J., and Hayden, M. R. (1999) *Lancet* **354,** 1341–1346
8. Remaley, A. T., Rust, S., Rosier, M., Knapper, C., Naudin, L., Broccardo, C., Peterson, K. M., Koch, C., Arnould, I., Prades, C., Duverger, N., Funke, H., Assman, G., Dinger, M., Dean, M., Chimini, G., Santamarina-Fojo, S., Fredrickson, D. S., Denefle, P., and Brewer, H. B., Jr. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12685–12690
9. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J.-C., Deleuze, J.-F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) *Nat. Genet.* **22,** 352–355
10. Klucken, J., Büchler, C., Orsó, E., Kaminski, W. E., Porsch-Özcürümez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R., and Schmitz, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 817–822
11. Kaminski, W. E., Piehler, A., and Schmitz, G. (2000) *Biochem. Biophys. Res. Commun.* **278,** 782–789
12. Tanaka, A. R., Ikeda, Y., Abe-Dohmae, S., Arakawa, R., Sadanami, K., Kidera, A., Nakagawa, S., Nagase, T., Aoki, R., Kioka, N., Amachi, T., Yokoyama, S., and Ueda, K. (2001) *Biochem. Biophys. Res. Commun.* **283,** 1019–1025
13. Wang, N., Lan, D., Gerbod-Giannone, M., Linsel-Nitschke, P., Jehle, A. W., Chen, W., Martinez, L. O., and Tall, A. R. (2003) *J. Biol. Chem.* **278,** 42906–42912
14. Kojima, K., Abe-Dohmae, S., Arakawa, R., Murakami, I., Suzumori, K., and Yokoyama, S. (2001) *Biochim. Biophys. Acta* **1532,** 173–184
15. Arakawa, R., and Yokoyama, S. (2002) *J. Biol. Chem.* **277,** 22426–22429
16. Yokoyama, S., Tajima, S., and Yamamoto, A. (1982) *J. Biochem. (Tokyo)* **91,** 1267–1272
17. Tajima, S., Yokoyama, S., and Yamamoto, A. (1983) *J. Biol. Chem.* **258,** 10073–10082
18. Abe-Dohmae, S., Suzuki, S., Wada, Y., Aburatani, H., Vance, D. E., and Yokoyama, S. (2000) *Biochemistry* **39,** 11092–11099
19. Kishimoto, T., Soda, Y., Matsuyama, Y., and Mizuno, K. (2002) *Clin. Biochem.* **35,** 411–416
20. Tanaka, A. R., Abe-Dohmae, S., Ohnishi, T., Aoki, R., Morinaga, G., Okuhira, K., Ikeda, Y., Kano, F., Matsuo, M., Kioka, N., Amachi, T., Murata, M., Yokoyama, S., and Ueda, K. (2003) *J. Biol. Chem.* **278,** 8815–8819
21. Sasaki, M., Shoji, A., Kubo, Y., Nada, S., and Yamaguchi, A. (2003) *Biochem. Biophys. Res. Commun.* **304,** 777–782
22. Yamauchi, Y., Abe-Dohmae, S., and Yokoyama, S. (2002) *Biochim. Biophys. Acta* **1585,** 1–10
23. Wang, N., Chen, W., Linsel-Nitschke, P., Martinez, L. O., Agerholm-Larsen, B., Silver, D. L., and Tall, A. R. (2003) *J. Clin. Invest.* **111,** 99–107
24. Yamauchi, Y., Hayashi, M., Abe-Dohmae, S., and Yokoyama, S. (2003) *J. Biol. Chem.* **278,** 47890–47897
25. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993) *J. Biol. Chem.* **268,** 9194–9197
26. Orso, E., Broccardo, C., Kaminski, W. E., Böttcher, A., Liebisch, G., Drobnik, W., Götz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M.-F., Rothe, G., Lackner, K. J., Chimini, G., and Schmitz, G. (2000) *Nat. Genet.* **24,** 192–196
27. McNeish, J., Aiello, R. J., Guyot, D., Turi, T., Gabel, C., Aldinger, C., Hoppe, K. L., Roach, M. L., Royer, L. J., de Wet, J., Broccardo, C., Chimini, G., and Francone, O. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4245–4250
28. Lorkowski, S., Kratz, M., Wenner, C., Schmidt, R., Weitkamp, B., Fobker, M., Reinhardt, J., Rauterberg, J., Galinski, E. A., and Cullen, P. (2001) *Biochem. Biophys. Res. Commun.* **283,** 821–830
29. Wang, N., Silver, D. L., Thiele, C., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 23742–23747
30. Smith, J. D., Miyata, M., Ginsberg, M., Grigaux, C., Shmookler, E., and Plump, A. S. (1996) *J. Biol. Chem.* **271,** 30647–30655
31. Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) *J. Biol. Chem.* **34508–34511**
32. Santamarina-Fojo, S., Peterson, K., Knapper, C., Qiu, Y., Freeman, L., Cheng, J. F., Osorio, J., Remaley, A., Yang, X. P., Haudenschild, C., Prades, C., Chimini, G., Blackmon, E., Francois, T., Duverger, N., Rubin, E. M., Rosier, M., Denefle, P., Fredrickson, D. S., and Brewer, H. B., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7987–7992
33. Becq, F., Hamon, Y., Bajetto, A., Gola, M., Verrier, B., and Chimini, G. (1997) *J. Biol. Chem.* **272,** 2695–2699
34. Haidar, B., Denis, M., Krimbou, L., Marcil, M., and Genest, J., Jr. (2002) *J. Lipid Res.* **43,** 2087–2094
35. See, R. H., Caday-Malcolm, R. A., Singaraja, R. R., Zhou, S., Silverston, A., Huber, M. T., Moran, J., James, E. R., Janoo, R., Savill, J. M., Rigot, V., Zhang, L. H., Wang, M., Chimini, G., Wellington, C. L., Tafuri, S. R., and Hayden, M. R. (2002) *J. Biol. Chem.* **277,** 41835–41842
36. Broccardo, C., Osorio, J., Luciani, M. F., Schriml, L. M., Prades, C., Shulenin, S., Arnould, I., Naudin, L., Lafargue, C., Rosier, M., Jordan, B., Mattei, M. G., Dean, M., Denefle, P., and Chimini, G. (2001) *Cytogenet. Cell Genet.* **92,** 264–270
37. Assmann, G., von Eckardstein, A., and Brewer, H. B., Jr. (2001) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. C., Valle, D., and Sly, W. S., eds) 8th Ed., pp. 2937–2960, McGraw-Hill, New York
38. Christiansen-Weber, T. A., Voland, J. R., Wu, Y., Ngo, K., Roland, B. L., Nguyen, S., Peterson, P. A., and Fung-Leung, W. P. (2000) *Am. J. Pathol.* **157,** 1017–1029

Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 8, Issue of February 20, pp. 6217–6220, 2004
Printed in U.S.A.

Phosphorylation and Stabilization of ATP Binding Cassette Transporter A1 by Synthetic Amphiphilic Helical Peptides*

Received for publication, December 19, 2003
Published, JBC Papers in Press, December 29, 2003,
DOI 10.1074/jbc.C300553200

Reijiro Arakawa‡§, Michi Hayashi‡, Alan T. Remaley¶, Bryan H. Brewer¶, Yoshio Yamauchi‡‖, and Shinji Yokoyama‡**

From the ‡Biochemistry, Cell Biology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8 601, Japan, the §Research and Development Division, Grelan Pharmaceutical Co., Ltd., Sakaecho 3-4-3, Hamura, Tokyo 205-0002, Japan, and the ¶National Institutes of Health Molecular Disease Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

To investigate structural requirement of helical apolipoprotein to phosphorylate and stabilize ATP-binding cassette transporter A1 (ABCA1), synthetic peptides (Remaley, A. T., Thomas, F., Stonik, J. A., Demosky, S. J., Bark, S. E., Neufeld, E. B., Bocharov, A. V., Vishnyakova, T. G., Patterson, A. P., Eggerman, T. L., Santamarina-Fojo, S., and Brewer, H. B. (2003) *J. Lipid Res.* 44, 828–836) were examined for these activities. L37pA, an L amino acid peptide that contains two class-A amphiphilic helices, and D37pA, the same peptide with all D amino acids, both removed cholesterol and phospholipid from differentiated THP-1 cells more than apolipoproteins (apos) A-I, A-II, and E. Both peptides also mediated lipid release from human fibroblasts WI-38 similar to apoA-I. L2D37pA, an L-peptide whose valine and tyrosine were replaced with D amino acids also promoted lipid release from WI-38 but less so with THP-1, whereas L3D37pA, in which alanine, lysine, and asparatic acid were replaced with D amino acids was ineffective in lipid release for both cell lines. ABCA1 protein in THP-1 and WT-38 was stabilized against proteolytic degradation by apoA-I, apoA-II, and apoE and by all the peptides tested except for L3D37pA, and ABCA1 phosphorylation closely correlated with its stabilization. The analysis of the relationship among these parameters indicated that removal of phospholipid triggers signals for phosphorylation and stabilization of ABCA1. We thus concluded that an amphiphilic helical motif is the minimum structural requirement for a protein to stabilize ABCA1 against proteolytic degradation.

Release of cellular cholesterol is one of the essential events of its homeostasis for the cells and also for the whole body, since cholesterol is catabolized only in the a few certain organs, mainly in the liver for the conversion to bile acids and in very limited amount in steroidogenic organs. This reaction is mediated by two distinct mechanisms: 1) nonspecific physicochemical exchange with cell surface and extracellular cholesterol acceptors and 2) by a specific mechanism for helical apolipoproteins such as apolipoprotein (apo)[1] A-I, which remove cellular phospholipid and cholesterol to generate new high density lipoprotein (HDL) (1). The latter reaction is known to be mediated by the ATP-binding cassette transporter A1 (ABCA1) (2–4) and is considered a rate-limiting step in regulating of the HDL concentration in blood plasma (5–7). It is therefore of clinical importance as it may influence this strong negative risk factor for atherosclerotic vascular disease.

ABCA1 is degraded by calpain, and it appears to be one of the major mechanisms for regulation of its cellular level, and helical apolipoproteins protect ABCA1 against this degradation (8, 9). Helical apolipoproteins interact with ABCA1 and generate new HDL by removing cellular lipid, ABCA1 is phosphorylated and stabilized by a mechanism involving protein kinase C that is presumably activated by diacylglycerol generated by replenishment reaction for the removal of sphingomyeline (10). Stabilization of ABCA1 is observed not only with apoA-I but also with other apolipoproteins capable of lipid removal such as apoA-II or apoE (8, 9), which is consistent with the hypothesis that the removal of lipid triggers the signaling for ABCA1 stabilization rather than some other type apolipoprotein interaction with the cells.

Synthetic peptides that mimic amphiphilic helical segments of apolipoproteins were demonstrated to reproduce the lipid removal reaction by apolipoproteins, indicating that structural requirement for apolipoproteins to carry out this reaction is not highly specific (11, 12). Furthermore, both L and D stereoisomer forms of the peptides were equally efficient in promoting lipid release (13). This is similar to the structural requirements described for reactions of lecithin:cholesterol acyltransferase (14) and cholesteryl ester transfer protein (15).

In this study, we therefore examined the phosphorylation and stabilization of ABCA1 with series of synthetic peptides that mimic amphihphilic helical proteins (13). In addition, L or D stereoisomers, as well as peptides with a mixture of L and D amino acids were tested (13). The results are consistent with a model whereby peptides with an amphipathic helical structure that are competent in promoting lipid efflux from cells also promote the phosphorylation and stabilization of ABCA1.

EXPERIMENTAL PROCEDURES

Materials—ApoA-I and apoA-II were isolated from HDL fraction prepared from fresh human plasma as described elsewhere (16). Recombinant human apoE3 was a generous gift from Mitsubishi Pharma Corp. (Yokohama, Japan). ApoC-III was prepared from human plasma as described previously (17). The L37pA peptide (DWLKAFYDKVAEKLKEAFPDWLKAFYDKVAEKLKEAF) was synthesized by a solid phase procedure as previously described with all L amino acids (13). The D37pA peptide was synthesized with the same sequence but with D amino acids (13). Valine and tyrosine were replaced with D amino acids for the L2D37pA peptide, and alanine, lysine, and asparatic acid were replaced with D amino acids for the L3D37pA peptide (13).

* This work was supported by a grants-in-aids from Ministries of Health, Welfare and Labor of Japan, and of Education, Science, Culture and Sports of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ A Research Fellow of the JSPS for Young Scientists.

** To whom correspondence should be addressed. Tel.: 81-52-853-8139; Fax: 81-52-841-3480; E-mail: syokoyam@med.nagoya-cu.ac.jp.

[1] The abbreviations used are: apo, apolipoprotein; HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; PMA, phorbol 12-myristate 13-acetate.

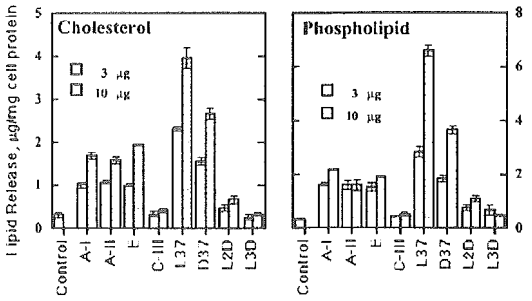

FIG. 1. **Lipid release from the differentiated THP-1 cells.** The cells were differentiated by the PMA treatment for 72 h and then incubated with the indicated apolipoproteins or synthetic peptides, either 3 or 10 μg in 1 ml of the culture medium. After the 24-h incubation, the medium was recovered and cholesterol and choline-phospholipid were measured.

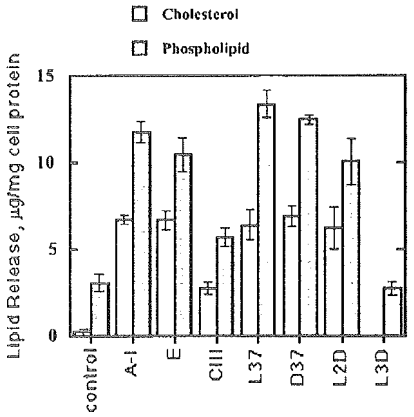

FIG. 2. **Lipid release from WI-38 cells.** The cells were incubated with apoA-I or the indicated synthetic peptides, 10 μg each, in 1 ml of medium. After 24 h, cholesterol and choline-phospholipid in the medium were measured. Choline-phospholipid detected in the absence of apolipoproteins or peptides was previously shown to be mostly lysophosphatidylcholine (18).

Cellular Lipid Release—Human monocytic cell line cells, THP-1, differentiated with phorbol 12-myristate 13-acetate (PMA) for 72 h (8), and human lung fibroblasts, WI-38, pretreated with 9-*cis*-retinoic acid for 18 h, were maintained as described previously (10, 18). The cells were incubated with apolipoproteins or peptides for 24 h to induce the release of cellular lipid. Cholesterol and choline-phospholipid in the medium were measured by a respective enzymatic assay system (19).

Immunobloting, Pulse Labeleing, and Phosphorylation of ABCA1—The cells were incubated with apolipoproteins or peptides for 24 h, and ABCA1 in the cells was analyzed by immunoblotting by using an antibody against the C-terminal peptide of human ABCA1 according to a previous method (20). For estimation of the rate of decay of ABCA1, the cells were pulse labeled with ^{35}S-labeled amino acids for 30 min and incubated with apolipoproteins or peptides prior to the analysis by electrophoresis-autoradiography of the immunoprecipitated fraction with the anti-ABCA1 antibody as described previously (8). Phosphorylation of ABCA1 was examined by incubating the cells with apolipoproteins of peptides for 1 h in the presence of ^{32}P prior to the analysis by electrophoresis-autoradiography of the fraction immunoprecipitated with the anti-ABCA1 antibody, as described previously (10).

RESULTS AND DISCUSSION

Lipid release was examined from differentiated THP-1 cells by apoA-I, apoA-II, apoE, and apoC-III and for the peptides L37pA, D37pA, L2D37pA, and L3D37pA (Fig. 1). ApoA-I, apoA-II, and apoE showed similar lipid release activity on a per weight basis, with approximately a 1:1 mass ratio of cholesterol to choline-phospholipid. ApoC-III, however, exhibited only slight activity for lipid release. The peptide L37pA resulted in approximately a 4-fold more choline-phospholipid release and a 2-fold more cholesterol release compared with the apolipoproteins when used at the same protein mass concentration. Similarly, the D37pA peptide also promoted more cholesterol and phospholipid release than the apolipoproteins, although it was slightly less effective than the L37pA peptide. L2D37pA removed more lipid than apoC-III, but it was much less effective than the other apolipoproteins and the L37pA and D37pA. L3D37pA was completely ineffective in promoting lipid removal from cells, as described previously (13).

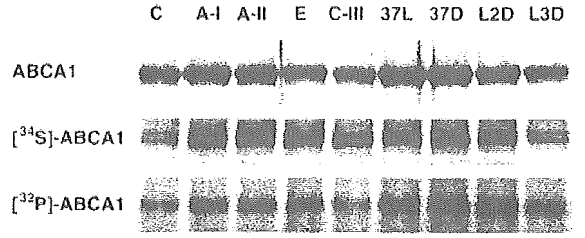

FIG. 3. **Analysis of ABCA1 in THP-1 cells.** THP1 cells were differentiated by the PMA treatment for 72 h and incubated with the indicated apolipoproteins or peptides, 10 μg/ml. The protein level of ABCA1 was analyzed by immunoblotting by using an antibody against the C terminus of ABCA1 after the 24-h incubation. To examine the rate of degradation, ABCA1 was pulse-labeled with ^{34}S-labeled amino acid mixture for 30 min, and the cells were incubated with the apolipoproteins or the synthetic peptides for 4 h before ABCA1 was immunoprecipitated with the antibody and analyzed by electrophoresis and autoradiography. To study phosphorylation of ABCA1, the cells were incubated with the apolipoproteins or the synthetic peptides in the presence of ^{32}P for 1 h, and ABCA1 was analyzed by electrophoresis and autoradiography after immunoprecipitation.

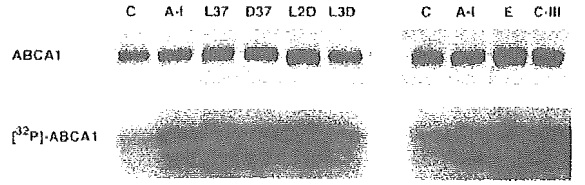

FIG. 4. **Analysis of ABCA1 in WI-38 cells.** The cells were treated with apoA-I or with the indicated synthetic peptides, 10 μg/ml, for the analysis of cellular level and phosphorylation of ABCA1, in the same manner as the experiments for THP-1 cells described in the legend for Fig. 3.

Similar lipid release experiments were carried out with WI-38 cells (Fig. 2). This cell line releases relatively high amount of choline-phospholipid at base line without any apolipoproteins or any other "lipid acceptor" in the media (18). We have shown previously that this is mostly due to lysophosphatidylcholine release (18). Interestingly, unlike THP-1 cells, L37pA and D37pA showed similar lipid releasing activity to that by apoA-I. Furthermore, in contrast to THP-1 cells, L2D37pA also demonstrated equivalent capability of lipid removal compared with apoA-I, apoE, L37pA, and D37pA. L3D37pA, however, was incapable of removing lipid from either WI-38 cells (Fig. 2) or THP-1 cells (Fig. 1).

ABCA1 in THP-1 cells was analyzed by immunobotting for its protein level, by protein pulse labeling for the rate of decay, and by using ^{32}P labeling for monitoring its state of phosphorylation (Fig. 3). Increase in the protein level of ABCA1 was observed in differentiated THP-1 cells after treatment with apoA-I, apoA-II, and apoE, but not so by apoC-III. L37pA and D37pA also markedly increased ABCA1 protein level. L2D37pA appeard to be slightly less effective in stabilizing ABCA1, and L3D37pA had no effect on the ABCA1 protein levels. The increase of ABCA1 protein was parallel with retardation of its decay, as indicated by retention of radioactivity in the protein after pulse labeling of the cells with ^{32}S-labeled

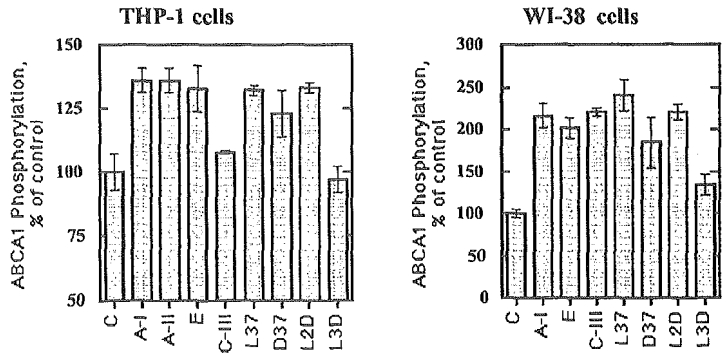

FIG. 5. **Extent of phopshorylation of ABCA1 standardized for the protein level.** Density of the bands by immunoblotting analysis and autoradiogram of the phosphorylated ABCA1 were quantitated by digital scanning in an Epson GT9500, to estimate the extent of its phosphorylation. The results are expressed as percentage to control (*C*, without apolipoprotein/peptide). Data represents mean ± S.E. of at least three independent experiments.

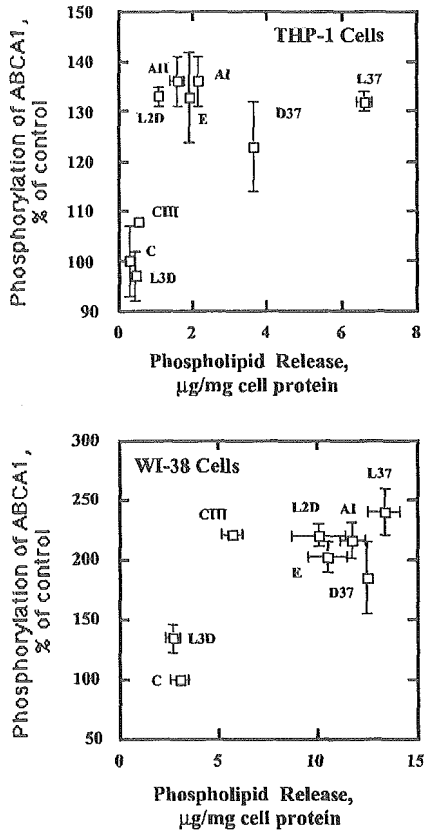

FIG. 6. **Relationship of ABCA1 phosphorylation to the removal of choline-phospholipid.** The data used in Fig. 5 were plotted the data used in Figs. 1 and 2 (for 10 μg/ml apolipoproteins or peptides), for THP-1 cells and WI-38 cells, respectively.

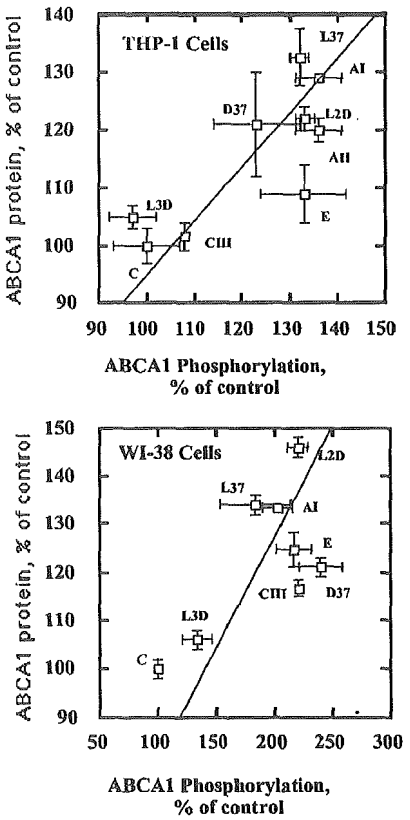

FIG. 7. **Relationship of ABCA1 protein level to the extent of phosphorylation of ABCA1.** The data used in Fig. 5 were plotted for THP-1 cells and WI-38 cells. *Lines* indicate a least square linear regression lines.

amino acids. Thus, stabilization of ABCA1 by helical apolipoproteins was reproduced with the use of synthetic amphiphilic helical peptides that are also capable of removing cellular lipid. Phosphorylation of ABCA1 was enhanced by stimulating the cells with apoA-I, apoA-II, apoE, L37pA, and D37pA. ABCA1 in WI-38 was also analyzed in a similar manner (Fig. 4). Increase of ABCA1 was demonstrated with apoA-I, apoE, L37pA, D37pA, and L2D37pA, and also with apoC-III but to less extent. Phosphorylation of ABCA1 was also shown with these apolipoproteins and peptides. Neither protein increase nor phosphorylation of ABCA1 was observed with L3D37pA. Fig. 5 shows the extent of ABCA1 phosphorylation standardized by protein level for THP-1 and WI-38 cells.

Relationships among the various measured parameters was analyzed in Figs. 6–8. Fig. 6 shows the extent of ABCA1 phopshorylation as a function of phospholipid release by various apolipoproteins and their model peptides. It appears that there is a threshold for the effect of phospholipids release on ABCA1 phosphorylation. Phosphorylation of ABCA1 reaches a maximum at a phospholipid release of ~2 μg/mg cell protein and increases no further, which is consistent with a model whereby removal of phospholipid is a trigger for signal transduction to phopshorylate ABCA1 (10). Phosphorylation of ABCA1 in WI-38 cells (Fig. 6) also increased with phospholipids release, and a similar threshold effect was demonstrated. It should be noted that apoC-III removed a significant amount of phospholipid from WI-38 cells and induced a maximum phosphorylation of ABCA1, while it was inefficient in both reactions in THP-1 cells. Fig. 7 shows the relationship between phospho-