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Accumulation of RhoA, RhoB, RhoG, and Rac1 in Fibroblasts from Tangier Disease Subjects Suggests a Regulatory Role of Rho Family Proteins in Cholesterol Efflux

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Tangier disease (TD) is an inherited disorder of lipid metabolism characterized by very low high density lipoprotein (HDL) plasma levels, cellular cholesteryl ester accumulation and reduced cholesterol excretion in response to HDL apolipoproteins. Molecular defects in the ATP binding cassette transporter 1 (ABCA1) have recently been identified as the cause of TD. ABCA1 plays a key role in the translocation of cholesterol across the plasma membrane, and defective ABCA1 causes cholesterol storage in TD cells. However, the exact relationship of many of the biochemical and morphological abnormalities in TD to ABCA1 is unknown. Since small GTP-binding proteins are important regulators of many cellular functions, we characterized these proteins in normal and TD fibroblasts using the [α-^{32}P]GTP overlay technique and Western blotting of SDS and isoelectric focusing gels. Our results indicate that GTP-binding proteins of the Rho family (RhoA, RhoB, RhoG, Rac-1) are enriched in fibroblasts from TD patients. The accumulation of small G proteins may have potential implications for the TD phenotype and the regulation of cholesterol excretion in TD cells. © 2001 Academic Press

Key Words: **Tangier disease (TD); high density lipoprotein (HDL); familial HDL deficiency; apolipoprotein A-I (apo A-I); ATP binding cassette transporter 1 (ABCA-1); small GTP-binding proteins; Rho proteins; cholesterol efflux; Western blotting of isoelectric focusing gels; GTP overlay.**

High-density-lipoprotein (HDL) is thought to play an important role in so-called reverse cholesterol transport, the process by which the cholesterol is transported from peripheral cells to the liver, where it may be excreted in the form of bile salts. This process is thought to protect against the development of atherosclerosis. The cholesterol efflux process involves specific and unspecific mechanisms (1, 2). Specific cholesterol efflux from cells is mediated by HDL apolipoproteins; it involves the activation of cellular phospholipases and kinases and an active brefeldin-sensitive transport pathway (3–6). The specific apolipoprotein-dependent cholesterol efflux is severely impaired in subjects with homozygous Tangier disease (TD) (7–10), resulting in almost complete absence of circulating HDL, massive accumulation of cholesteryl esters in many tissues and an overall 4- to 6-fold increased cardiovascular risk (11–13).

By genetic mapping (14) and subsequent sequencing of candidate genes on DNA from Tangier patients, the ABCA1 transporter has recently been identified as the molecular defect in TD (15–18). The ATP binding cassette (ABCA) superfamily is comprised of a large group of evolutionarily conserved proteins present in prokaryotes and eukaryotes (19). These proteins function by coupling the hydrolysis of ATP to the transmembrane flux of a wide variety of substrates across organellar and plasma membranes. ABCA1 seems to be an essential cofactor for the translocation of cholesterol across the plasma membrane (20), and defective ABCA1 in TD causes impaired cholesterol efflux in response to apolipoproteins. However, the mechanism of ABCA1-dependent cholesterol excretion is not entirely clear (21, 22). Moreover, ABCA1 may play a regulatory role in other cellular processes such as phagocytosis and apoptosis (23), embryogenesis (23), anion flux (24) and interleukin secretion (25). On the

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other hand, a diverse variety of as yet unexplained cellular abnormalities have been described in TD cells such as reduced PLD activity (4), enhanced phospholipid turnover (26), lipid accumulation along the cytoskeleton (27), golgi hyperplasia (27) and growth retardation (28). The possible relationship between these abnormalities and deficiency of normal ABCA1 is not understood, but may involve small GTP-binding proteins, which regulate a diverse spectrum of intracellular processes (29). In this study, we characterized the presence of such proteins in fibroblasts of patients with TD.

EXPERIMENTAL DETAILS

Probands. The experiments were performed with fibroblasts from four normolipidemic healthy probands and two homozygous Tangier patients of the JS family (patient JS: 66 y, male; patient EG:63 y, female, sister of JS) whose clinical and biochemical manifestations including HDL deficiency, splenomegaly and lipid storage in various reticulo-endothelial tissues have been described in detail in previous reports (4, 7, 11, 12, 26–28, 30). This family was the first TD family identified in Germany in 1977 (11) and was the first TD kindred in whom impaired apolipoprotein-dependent cholesterol excretion was demonstrated (7).

Cell culture. Human skin fibroblasts cultured from biopsies of adult human hip skin were grown and maintained in DMEM containing 10% FCS, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO). Once separated, the dermis was cut into small pieces (0.5 mm on each side) and placed in a flask in DMEM. When these primary cultures were confluent they were expanded by passage. For experiments, cells between passage levels three and six were seeded in 35-mm culture dishes. At the state of near-confluency (after 6 to 10 days), the cell extracts were prepared (freshly for each experiment).

Sample preparation. All sample preparation procedures were carried out at 4°C, and all tubes and rubber policemen were precooled. The cells were chilled on ice for 1 h, rinsed three times with ice-cold phosphate-buffered saline (PBS, pH 7.4) and drained well. The cells were scraped with a soft rubber policeman, transferred to tubes in 5 ml PBS and centrifuged at 1200 rpm for 10 min. For one-dimensional isoelectric focusing (IEF) and two-dimensional gel electrophoresis the pellet was resuspended in IEF sample buffer (10 mM Tris (ultra pure), 1% sodium decylsulfate (Kodak, Stuttgart, Germany), pH-adjusted to pH 8.2 with concentrated HCl, 2% ampholytes, pH ranges 3–10 from Serva and 3–10.5 from Pharmacia, blended in a ratio of 1:1). For SDS electrophoresis, the pellet was dissolved in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerine, 10% β-mercaptoethanol and 2.3% SDS). The samples were transferred to Eppendorf tubes and broken by three cycles of rapid freezing and thawing in liquid nitrogen. 0.5% RNAse and 1% DNAse (from Sigma) were immediately added and mixed. The solution was incubated for 15 min at room temperature. Protein concentrations were measured according to the method of Lowry (31), using bovine serum albumin as the standard. For this purpose, samples of fibroblast extracts were precipitated with 10% trichloroacetic acid, residual acid was neutralized and the proteins were completely dissolved in an ultrasound bath (5 min, 4°C), followed by the Lowry procedure. Following protein determination, 5 μl β-mercaptoethanol plus 10 μl 80% succrose was added to each sample, the sample volume was filled up with the respective sample buffer to a total volume of 100 μl, and incubated for 1 h at room temperature, directly followed by electrophoresis.

Two-dimensional gel electrophoresis. Two-dimensional electrophoresis was carried out by the method of O'Farrell (32). The first dimension isoelectric focusing gel was composed of 9.5 M urea, 2% Nonidet P-40, 3.8% acrylamide, 0.2% *N,N′*-methylenebisacrylamide, and 2% ampholytes (pH range 3.5–10). Samples were focused for 5000 Vh followed by 800 V for 1 h. The gels were then extruded and incubated for 10 min in reducing sample buffer (0.05 M Tris–HCl, pH 6.8, 6 M urea, 30% glycine, 1% SDS, 0.25% dithiothreitol) and for additional 10 min in the same solution with 4.5% iodoacetamide instead of dithiothreitol The second dimension SDS gel electrophoresis was performed according to Laemmli (33) using a 13% acrylamide separation gel and a 3% acrylamide stocking gel. Molecular weight standards were run in a well that was fashioned in one side of the second dimension gel.

One-dimensional isoelectric focusing. In the experiments shown in Fig. 3, the fibroblast proteins were separated in 7.5% polyacrylamide gels containing 2% ampholytes, pH 3.5–10, and 8 M urea in a Bio-Rad Mini-Protean II electrophoresis unit. Unless otherwise indicated, 100 μg protein were loaded on each lane. Isoelectric focusing was performed at 200 V for 12 h at 4°C with 0.01 M H_3PO_4 as the anode solution and 0.02 M NaOH as the cathode solution, followed by a final focusing at 600 V for 3 h.

One-dimensional SDS gel electrophoresis. For SDS gel electrophoresis the samples (100 μg) were diluted with SDS sample buffer, 5 μl β-mercaptoethanol and 10 μl 80% succrose were added, the samples were heated at 95°C for 3 min and analysed by SDS slab-gel electrophoresis at 4°C and 100 V, with 15% acrylamide in the separation gel. The running buffer contains 25 mM Tris–HCl, pH 8.6, 0.2 M glycerine and 0.1% SDS.

Western blot analysis. For immunoblotting, separated proteins were transferred to a 0.45 μm pore-size nitrocellulose membrane (Optitran BA-85 S., Schleicher & Schuell, Dassel, Germany) in 20 mM Tris–HCl, pH 9.0, 150 mM glycine and 20% methanol at 0.5 mA for 70 min, using a Bio Rad electrophoresis cell. After transfer, the filter was then blocked with 5% (w/v) nonfat dried milk powder (blocking solution from Amersham) at 4°C overnight. The filter was subsequently washed three times in PBS (10 min per wash), followed by a 60 min incubation at room temperature with the primary antibody at the appropriate dilution (2×10^3 in most experiments): polyclonal anti-(rabbit IgG) Rac-1, anti-(rabbit IgG) RhoA, anti-(rabbit IgG) RhoB, anti-(rabbit IgG) RhoG, anti-(rabbit IgG) Rho-7, anti-(rabbit IgG) Rho-8, anti-(rabbit IgG) α-PAK or a monoclonal anti-(mouse IgG1) Pan-Arf antibody. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except anti Pan-Arf, which was obtained from Alexis Corporation (San Diego, CA). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham). Quantification was performed with a phosphoimager (BAS 1500, Raytest, Straubenhardt, Germany).

For IEF immunoblotting, the IEF gel was allowed to equilibrate for 30 min in a solution, which was prepared as follows: 4 ml of a 0.04 M morpholine/2% SDS solution (20 μl morpholine from ICN Biomedicals, Eschwege, Germany, 5.75 ml distilled water, 0.11 g SDS, pH-adjusted to pH 9.4 with concentrated HCl), plus 400 μl β-mercaptoethanol, plus 3 ml 80% succrose were filled up to 40 ml with distilled water. This solution (pH ~7.8) must be prepared freshly. Subsequent blotting was performed as described above.

GTP[α-^{32}P] and GTP [γ-^{35}S] overlay. For GTP[α-^{32}P] and GTP[γ-^{35}S] overlay we have modified a protocol based on the method of Lapetina and Reep (34). After the protein transfer nitrocellulose blots were incubated for 1 h at 20°C in a buffer containing 0.05% Tween 20, 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 5% bovine serum albumin (BSA). Then the nitrocellulose was incubated for 45 min at room temperature in a plastic foil in 20 ml binding-buffer composed of 20 mM Tris–HCl, pH 8.0, 0.15 M $MgCl_2$, 0.3% BSA, 2 mM dithiothreitol, 0.1% Nonidet P-40, 60μg/ml t-RNA (*E. coli*), 60 μCi GTP[α-^{32}P] or 100 μCi GTP[γ-^{35}S] (Amersham Pharmacia Biotech, Freiburg, Germany). After thorough bathing five times in 250

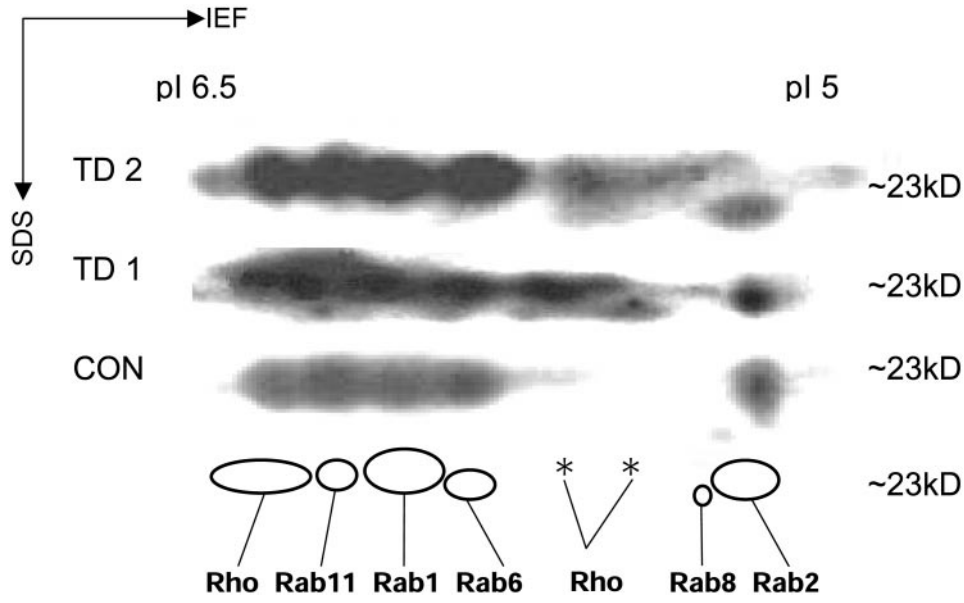

FIG. 1. [α-^{32}P]GTP overlay of small GTP-binding proteins of human skin fibroblasts from two homozygote Tangier patients (TD1, TD2) and one representative control (CON). The cellular extracts (100 μg) were separated by high-resolution two-dimensional gel electrophoresis as described under Experimental Details. The area of the blot shown here represents the 21- to 25-kDa range after SDS/15% gel electrophoresis. The directions of IEF and SDS electrophoresis are indicated. The pI range is shown at the top. The lower schematic image represents two groups of GTP-binding proteins, which have been found at identical electrophoretic positions in all cell types in a previous study (35) and served as internal positional markers. The proposed mapping according to Ref. 35 is shown; * represents putative GTP-binding proteins of the Rho family, which were mapped by anti-Rho antibodies exclusively.

ml washing solution (2% SDS, 0.5% Triton X-100, 0.5% BSA, 0.15 M NaCl; each washing step: 15 min at room temperature on a shaker), blots were air-dried and bound [^{32}P]/[^{35}S] radioactivity was visualized by autoradiography.

Sequencing of patient DNA. DNA of Tangier patients and controls was prepared and sequenced as described (14, 15). We selected primer sequences for amplification of the exons with inclusion of the intron/exon boundaries from intron sequences after establishing the boundaries by comparison of our genomic sequence with human ABCA1 mRNA. Genomic DNA from patients was amplified by PCR, and the products purified by PCR purification columns (Qiagen). Sequencing was done with the same primers as used in the PCR in conjunction with BigDye Terminator technology.

RESULTS

Cell extracts from control and TD cells were subjected to high-resolution two dimensional polyacrylamide gel electrophoresis followed by [α-^{32}P]GTP ligand binding. After renaturing transfer to nitrocellulose, the blots were overlaid with [α-^{32}P]GTP and the GTP-binding profiles were visualized by autoradiography. This method of GTP overlay after renaturing transfer is specific for small GTP-binding proteins, since the α-subunits of heterotrimeric G proteins are not visualized by this method (34). Using equal amounts of cell extracts, a group of closely adjacent GTP-binding proteins on the 2-D gel (in the pI range 5–7) was found to be markedly enriched in fibroblasts from the TD patients. The most striking difference was observed at the pI range 5–5.5, where we observed a dumbbell-shaped spot, which was almost in-visible in the controls (Fig. 1). By a combined strategy of overexpression and immunoblotting, Huber and coworkers mapped GTP-binding proteins of the Rho family at this electrophoretic position in Madin–Darby canine kidney cells (35). These proteins are barely detectable in normal human fibroblasts, probably because their concentration is below the lower detection limit of the GTP overlay technique. We therefore examined GTP-binding proteins of the Rho family in more detail using Western blot analysis with commercially available antibodies against small G proteins of the Rho/Rac family. Additionally, we performed Western blots with antibodies directed against Arf (another less closely related small GTPase) as control.

Since the area of interest was restricted to a relative narrow pH range, we compared the GTP-binding proteins using one-dimensional gels, which allow a direct comparison of control and TD cells on a single nitrocellulose sheet. Using SDS gel electrophoresis as separation method, all antibodies as well as the GTP overlay technique produced a single band on the SDS gels (Fig. 2), indicating that the vast majority of small G proteins in human fibroblasts have a similar molecular weight of approximately 21–25 kDa. Visualization of all small G proteins on SDS gels with the GTP overlay technique did not reveal a visible difference between control and TD cells (Fig. 2, top). Western blot analysis, by contrast, revealed a much higher signal in both TD cell

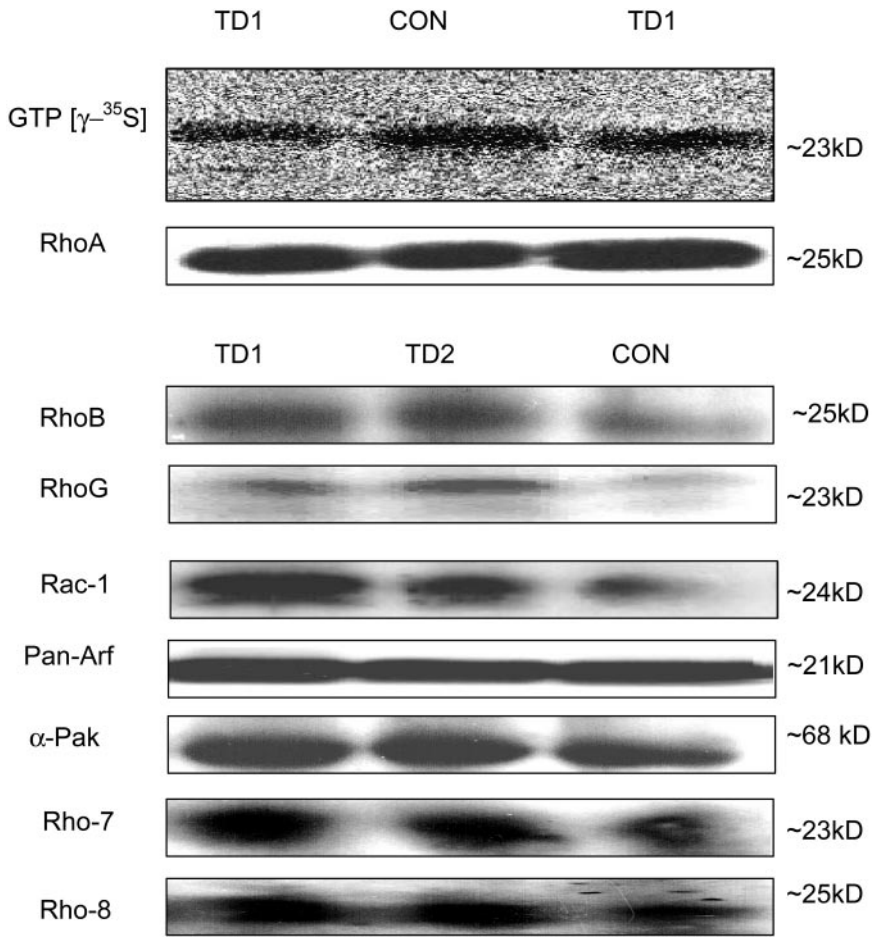

FIG. 2. Separation of cellular extracts by one-dimensional SDS gel electrophoresis. Cell extracts (100 μg) of human skin fibroblasts from two homozygote Tangier patients (TD1, TD2) and one representative control (CON) were separated by one-two-dimensional SDS/15% gel electrophoresis, followed by [γ-^{35}S]GTP overlay or by immunoblotting with antibodies against RhoA, RhoB, RhoG, Rac-1, Pan-Arf, α-Pak, Rho-7, or Rho-8.

lines when cell extracts were probed with antibodies directed against human RhoB, RhoG, or Rac-1. Extracts probed with antibodies against Arf, Rho-7, Rho-8, or against α-Pak, a downstream effector of Rac-1, did not differ between TD and control cells (Fig. 2). Extracts probed with anti RhoA gave a slightly enhanced signal in TD cells in some experiments.

Next, we separated the GTP-binding proteins by their charge and performed Western blot experiments following isoelectric focusing. For that purpose, we used an equilibration procedure that is advantageous to probe any proteins which are separable by charge rather than molecular weight. Protein variants produced by point mutations or posttranslational modifications can theoretically be recognized with this method, and the simultaneous separation of different cell extracts on the same gel allows a direct comparison, independent from gel to gel variations. As shown in Fig. 3, Western blotting of IEF gels revealed a high cross-reactivity of all commercially available antibodies used in this study. In accordance with the described results on SDS gels RhoB, RhoG, Rac-1, all immunologically related proteins and (to a lesser extent) RhoA were found to be at least twofold enriched in TD cells. The most striking difference was observed when the cell extracts were probed with anti-RhoG. When cell extracts were probed with this antibody, we found a protein with an apparent isoelectric point of ~5.7 that was almost not detectable in control cells under standard conditions (Fig. 3, third column).

This protein became visible in controls only at a 10-fold increased protein concentration (Fig. 3, fourth column). With anti-Arf, anti-α-Pak, or antibodies of other Rho family members the isoelectric focusing pattern was not significantly different between control and TD cells.

The described data suggested that the relative content of G proteins is different in control and TD cells, respectively. However, we found no indication for a structural defect of a G protein. The molecular defect in some other TD families was ascribed to ABCA1 (15–18). Sequencing ABCA1 from DNA of patient TD1 was

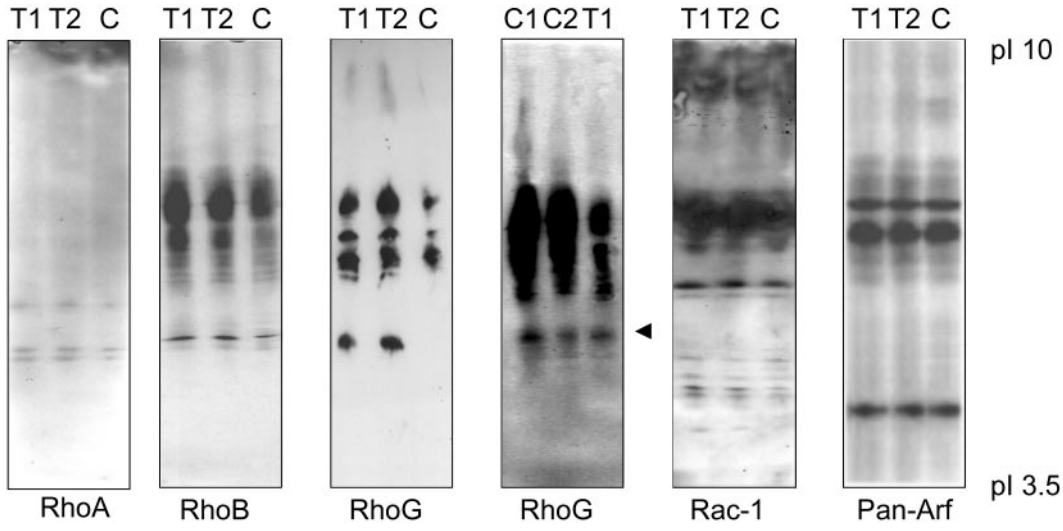

FIG. 3. Separation of cellular extracts by one-dimensional isoelectric focusing. Cell extracts (100 μg) of human skin fibroblasts from two homozygote Tangier patients (T1, T2) and one representative control (C) were separated by one-two-dimensional IEF, followed by immunoblotting with antibodies against RhoA, RhoB, RhoG, Rac-1, or Pan-Arf. In one experiment (column 4), separated extracts from two control cell lines (C1 and C2) were 10-fold enriched, relative to the cell extract from patient TD1 (C1 and C2, 200 μg; T1, 20 μg). The GTP-binding protein indexed by an arrow was markedly enriched in TD fibroblasts.

performed as previously described (15). We discovered two variants affecting the amino acid sequence and some silent variants. The relevant mutation was an asparagine to serine amino acid substitution in exon 19 of both homozygous individuals (AAT–AGT; amino acid position 935 of the primary translation product). This mutation was also discovered in an unrelated Spanish Tangier homozygote, but not in any normal individual (Rust *et al.*, unpublished results). A second amino acid substitution was found in exon 18 of both patients (ATA–ATG, I883 M of the primary translation product) (Fig. 4). This mutation has previously been suggested to be causative for TD in another kindred (TD4 in (17)) because it is localized in a putative ABCA1 phosphorylation site. However, this variant turned out to be a common polymorphism and was not related to reduced HDL cholesterol levels in the general population. In addition some silent DNA variants were found, and at two common polymorphic sites (arginine/lysine) in exons 7 and 35 with no significant influence on HDL-cholesterol, the patient was homozygous for lysine in exon 7 and arginine in exon 35, respectively.

DISCUSSION

In this study, we demonstrated the accumulation of GTP-binding proteins of the Rho family in fibroblasts from homozygous TD patients. This finding was verified by three different methods: the [^{32}P]GTP overlay technique using 2D polyacrylamide gels, Western blotting using one-dimensional SDS electrophoresis and Western blotting using one-dimensional IEF. Both RhoB, RhoG, Rac-1, and (to a lesser extent) RhoA were found to be enriched in TD cells. The most striking difference was observed on an as yet unidentified 23 kD G protein that was immunologically closely related to RhoG and was several-fold enriched in TD fibroblasts. Future work must show whether this protein is

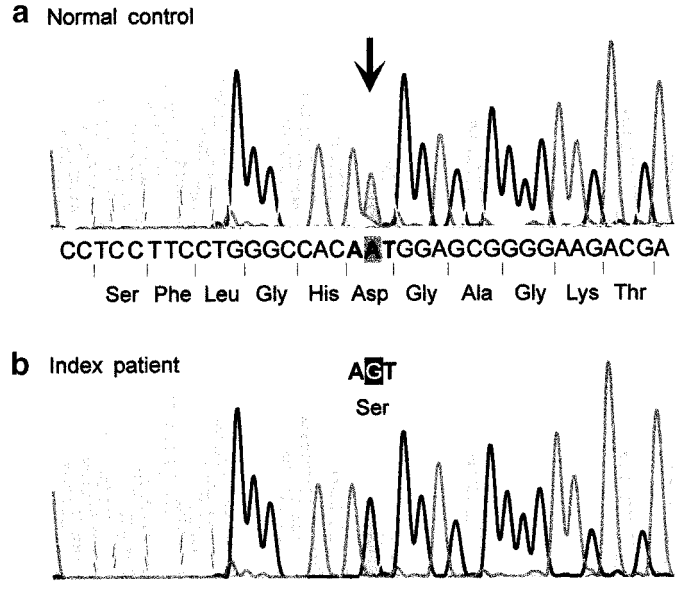

FIG. 4. Homozygous point mutation AAT–AGT (asparagine–serine, N935S). (a) Sequence of normal control, (b) sequence of the Tangier patient TD1. From ABI-377 sequencing files raw data were extracted using Chromas 1.45 software and these data were finally transferred to Micrographics Designer 7.1. To emphasize the variant base position (arrow in a) the area under the curve was filled.

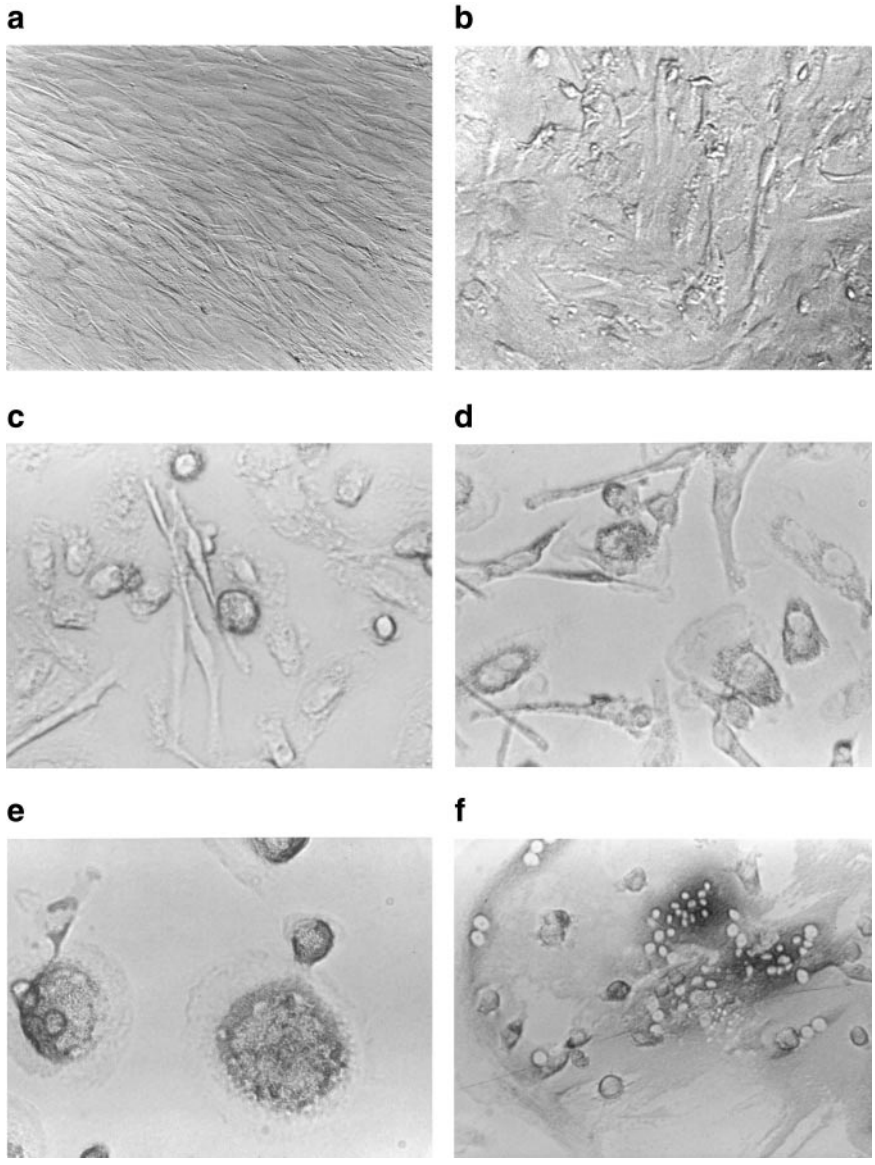

FIG. 5. Photomicrographs of control and Tangier cells. Control fibroblasts (a) are spindle-shaped and show an uniform, parallel alignment with minimal overlapping of cells. Fibroblasts from the homozygous TD patient TD1 (b) are characterized by a more asynchronous cell growth, compared to the age-matched control under identical culture conditions. Monocyte-derived macrophages from the TD1-patient (c, d, <10-day-old cultures; e, f, >10-day-old cultures) show an enhanced formation of membrane protrusions, especially in higher stages of maturation, leading to enhanced self-phagocytosis and giant cell formation (f). a, b, f × 150; c, d, e × 300.

one of the other 14 as yet known Rho family proteins or a totally new G protein. The relative cellular amount of other proteins of the Rho family (Rho-7, Rho-8) and of G proteins of other families such as Arf was not significantly different in TD and control cells. Also α-Pak, a downstream effector of Rac1 that controls mitogenic signals via a MAP kinase dependent pathway (36), was normal in TD cells.

The molecular defect of the here described TD kindred was localized to ABCA1, excluding the possibility that the TD phenotype in this kindred was caused by a different gene. Moreover, we found no indication for a structural defect of a small G protein. Two possibilities may account for the cellular accumulation of G proteins in TD cells. First, lipid accumulation in TD cells is associated with accumulation of small G proteins that are intimately involved in intracellular lipid transport. Deficient translocation of a small G protein from intracellular sites to the plasma membrane could then contribute to other characteristics of these cells such as receptor-associated abnormalities in cell signalling. Second, enhanced expression of small G proteins may have occurred secondarily, possibly as the result of an adaptative mechanism.

The Ras-related small GTP-binding proteins are molecular switches that are involved in diverse cellular

events, including cell signalling, proliferation, cytoskeletal organization, and secretion. Small GTP-binding proteins of the Rho family play an important role in transducing signals linking plasma membrane receptors to the organization of the cytoskeleton (37). They affect cell morphology by controlling the formation of actin-dependent structures and also participate in many aspects of the signaling of cell growth and differentiation. A diverse variety of abnormalities have been found in TD cells that may be related to the here described accumulation of Rho proteins. For example, we have shown in an earlier study that the receptor-dependent activation of PLD is impaired and delayed in TD fibroblasts, whereas the enzyme itself is not defective (4). Rho small GTPases are important cofactors for the activation of phospholipase D (38), and defective cellular location or defective coupling of a Rho protein with a plasma membrane receptor could cause defective PLD activation subsequent to agonist binding. Moreover, the Rho GTPases play an important role in transducing signals linking plasma membrane receptors to the organization of the cytoskeleton. Of relevance in this context may be the fact that Tangier fibroblasts accumulate lipids along the cytoskeleton, associated with Golgi hyperplasia (27), suggesting the possible involvement of the cytoskeleton and Rho GTPases in lipid excretion.

It is possible that enhanced synthesis of Rho family proteins partly compensates for ABCA1 deficiency in TD. For example, experiments with the inhibitor brefeldin suggest that vesicular trafficking is involved in cholesterol excretion (6). Moreover, recent findings suggest that more than one specific cholesterol excretion pathways may exist (39, 40). Upregulation of such an alternative cholesterol excretion pathway could explain why TD cells have a resting cholesterol efflux capacity (7) and why not all TD patients develop premature atherosclerosis (30). Moreover, ABCA transporters as well as Rho proteins are required for normal cell growth and development (37, 41). HDL-inducible cell signalling involved in mitogenesis is normal in TD fibroblasts (42). However, TD fibroblasts are characterized by a reduced growth rate (28), and the *in vitro* cell growth appears more irregular and asynchronous in cultivated TD fibroblasts (Fig. 5b). This is of interest insofar as Rac-1 and RhoG modulate the saturation density to which the cells grow (41). In addition, proteins of the Rho/Rac family are regulators of oxidative processes (43). Enhanced rather than decreased levels of oxidation products were observed in TD plasma (44). It is therefore of interest that oxidative cholesterol modification seems to be a key step in alternative cholesterol excretion pathways (40). Finally, both ABCA1 and Rho proteins are intimately involved in regulation of engulfment, which is the phagocytic clearance of cell corpses generated by apoptosis (23, 45). Despite a functional ABCA1 defect also this process appears to be rather enhanced than suppressed in cells from the TD kindred described here (Fig. 5). In conclusion, the further characterization of G proteins in TD may improve our understanding of the TD phenotype and of the cellular function of ABCA1 and its interaction with other cell proteins.

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