Localization of StarD5 cholesterol binding protein

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Abstract   Human StarD5 belongs to the StarD4 subfamily of START (for steroidogenic acute regulatory lipid transfer) domain proteins. We previously reported that StarD5 is located in the cytosolic fraction of human liver and binds cholesterol and 25-hydroxycholesterol. After overexpression of the gene encoding StarD5 in primary rat hepatocytes, free cholesterol accumulated in intracellular membranes. These findings suggested StarD5 to be a directional cytosolic sterol transporter. The objective of this study was to determine the localization of StarD5 in human liver. Western blot analysis confirmed StarD5's presence in the liver but not in human hepatocytes. Immunohistochemistry studies showed StarD5 localized within sinusoidal lining cells in the human liver and colocalized with CD68, a marker for Kupffer cells. Western blot analyses identified the presence of StarD5 in monocytes and macrophages as well as mast cells, basophils, and promyelocytic cells, but not in human hepatocytes, endothelial cells, fibroblasts, osteocytes, astrocytes, or brain tissue. Cell fractionation and immunocytochemistry studies on THP-1 macrophages localized StarD5 to the cytosol and supported an association with the Golgi.▪ The presence of this cholesterol/25-hydroxycholesterol-binding protein in cells related to inflammatory processes provides new clues to the role of this protein in free sterol transport in the cells and in lipid-mediated atherogenesis.—Rodriguez-Agudo, D., S. Ren, P. B. Hylemon, R. Montañez, K. Redford, R. Natarajan, M. A. Medina, G. Gil, and W. M. Pandak. **Localization of StarD5 cholesterol binding protein.** *J. Lipid Res.* 2006. **47:** 1168–1175.

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StarD5 belongs to the StarD4 subfamily, a subfamily of the steroidogenic acute regulatory lipid transfer (START) domain superfamily of proteins, which are involved in several pathways of intracellular trafficking and metabolism of cholesterol (1–3). The ~210 amino acid START domain contains a binding pocket, which determines the ligand binding specificity and function of each START domain protein (4). Although the functions of some START

domain proteins, such as StarD1, PCTP/StarD2, and MLN64/StarD3, have been studied extensively (5–11), the roles and characteristics of the proteins of the StarD4 subfamily have remained uncertain.

The StarD4 subfamily includes proteins StarD4, StarD5, and StarD6 (12). StarD4 and StarD5 are widely expressed, with the greatest levels of mRNA expression in the liver, whereas StarD6 appears limited to the testis (12). In contrast to other START domain proteins, such as StarD1 and MLN64, no members of the StarD4 subfamily have known N-terminal targeting sequences to direct these proteins to specific subcellular compartments; therefore, they are predicted to be cytoplasmic proteins (12, 13).

The localization of StarD6 to the testis was confirmed recently. This protein appears to play a role during germ cell maturation in adult testis (14). Recent studies by Soccio et al. (12) have confirmed previous observations on the sterol-mediated regulation of StarD4 expression. These new studies are consistent with the regulation of StarD4 by the sterol-regulatory element binding protein 2 and with the ability of StarD4 to transfer cholesterol in steroidogenesis assays after its transfection in COS-1 cells (15). The cellular localization of StarD4 remains uncertain, although recent studies have localized a green fluorescent protein-StarD4 fusion protein to the cytoplasm and nucleus of HeLa cells (16).

StarD5 mRNA expression has been reported to be induced in response to endoplasmic reticulum (ER) stress in free cholesterol-loaded mouse macrophages or in NIH-3T3 cells treated with tunicamycin, thapsigargin, or brefeldin A (15). This difference in the regulation of StarD4/StarD5 indicates putative different roles in cholesterol metabolism for each protein. StarD5 has been shown to be able to transfer cholesterol in steroidogenesis assays after its transfection in COS-1 cells (15). Previously, we had shown that StarD5 is able to selectively bind cholesterol and the potent regulatory oxysterol, 25-hydroxycholesterol (17). Furthermore, we confirmed the presence of StarD5 protein in human liver and found it to be localized to the cytosolic fraction (17). Expression of the gene

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encoding StarD5 in primary rat hepatocytes led to a marked increase in microsomal free cholesterol (17). These data suggested that StarD5 may be a cytosolic sterol carrier protein.

The objective of this study was to determine the liver cells that express StarD5 and its subcellular localization and to gain insight into its function. Whereas our previous studies showed the presence of StarD5 protein in human liver (17), no StarD5 protein was detected in isolated hepatocytes. This study shows that StarD5 is localized within the Kupffer cells in the liver. Western blot analysis also demonstrated the presence of StarD5 in macrophages, monocytes, promyelocytic cells, mast cells, and basophils. Furthermore, immunocytochemistry on macrophages localized StarD5 in the cytosol and supported an association with the Golgi. These findings, coupled with the observation of increased microsomal cholesterol accumulation in hepatocytes with increasing StarD5 expression (17), support the idea that StarD5 is a cytosolic sterol carrier to the Golgi.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibody against human StarD5 was obtained as described previously (17), monoclonal mouse anti-human macrophage CD68 was purchased from Serotec (Oxford, UK), and monoclonal mouse anti-rat GM130 was purchased from BD Biosciences (San Jose, CA). Secondary antibodies Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 transferrin from human serum, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR). Filipin III for staining of free cholesterol and Histopaque 1077 for isolation of mononuclear cells from blood were purchased from Sigma (St. Louis, MO). EDTA solution, pH 8.0, for heat-induced epitope retrieval was purchased from Zymed (San Francisco, CA). Human liver sections (formalin-fixed and embedded in paraffin), human liver tissue, primary human hepatocytes, freshly isolated human hepatocyte suspensions, and non-parenchymal cells from human liver were provided by the Liver Tissue Procurement and Distribution System (N01-DK-9-2310). Normal goat serum was purchased from Jackson Immunoresearch (West Grove, PA). Protein determinations were carried out with the Bio-Rad Miniprotein Assay from Bio-Rad (Hercules, CA).

Cell cultures

Human THP-1 monocytes, HepG2 hepatocellular carcinoma cells, HL-60 promyeloblasts, U2-OS osteosarcoma, and HT-29 colon adenocarcinoma cell lines were purchased from the American Type Culture Collection and maintained according to the supplier's protocols. Human KU 812-F myelogenous leukemia lymphoblast cells were supplied by the European Collection of Animal Cell Cultures and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. Human HMC-1 mast cells were kindly supplied by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Human umbilical vein endothelial (HUVE) cells were isolated from human umbilical cords by collagenase digestion (18) and were maintained in Medium 199 containing HEPES (10 mM), L-glutamine (2 mM), and heparin (10 mg/ml) supplemented with 3 mg/l endothelial cell growth supplement

(Sigma) and 20% FBS. Primary human fibroblasts were kindly supplied by Dr. Dorne Yager (Virginia Commonwealth University) and were maintained in DMEM supplemented with 10% FBS. Samples of human astrocytes and brain tissue were supplied by Dr. H. Fillmore (Virginia Commonwealth University). Primary human monocytes were isolated from sodium EDTA-treated blood obtained from a healthy volunteer. The blood was layered on Histopaque 1077 (1:1) and centrifuged at 400 *g* for 30 min at room temperature. The resulting mononuclear-enriched layer was collected and resuspended in 2 volumes of sterile PBS, pH 7.4. The cells were pelleted by centrifugation at 250 *g* for 10 min at room temperature and then plated in RPMI-1640 medium minus 2-mercaptoethanol supplemented with 10% autologous serum at 37°C. After 3 h, nonadherent cells were removed by washing with sterile PBS, pH 7.4, and the remaining adherent monocyte-enriched cells were grown for 2 days at 37°C before subsequent analysis.

Western blot analyses for StarD5

Immunoblottings with polyclonal StarD5 antibody were performed as described previously (17).

Immunofluorescence microscopic detection of StarD5 and CD68 in liver sections

Human liver sections were deparaffinized in *o*-xylene and then rehydrated by passage through a graded series of ethanol and distilled water. CD68 antigen was retrieved by heating the slides in EDTA solution, pH 8.0, for 20 min. Blocking was accomplished by incubation with 5% normal goat serum in PBS containing 0.05% Tween-20 for 16 h at 4°C. For interaction with primary antibodies, sections were incubated with 2.5% normal goat serum in PBS/0.05% Tween-20 containing StarD5 antibody (dilution, 1:400) or CD68 antibody (dilution, 1:100) for 45 min at 37°C in an incubator. After the sections were washed in PBS/0.05% Tween-20 (3 × 20 min), the bound primary antibodies were visualized with Alexa Fluor 568 goat anti-rabbit IgG (for StarD5) or Alexa Fluor 488 goat anti-mouse IgG (for CD68). Sections were then washed in PBS/0.05% Tween-20 (3 × 20 min). DNA was stained with DAPI for 5 min at room temperature, and after washing in PBS, the slides were mounted with a coverslip and viewed with a Zeiss LSM 510 Meta confocal microscope. Control experiments were performed in the absence of the primary antibodies or with the StarD5 preimmune serum.

Immunofluorescence microscopic detection of StarD5 in THP-1 macrophages

THP-1 monocytes were differentiated to macrophages by adding 100 nM phorbol 12-myristate 13-acetate. Macrophages on coverslips were washed with PBS and fixed with PBS/3.7% formaldehyde for 30 min at 4°C and then rinsed three times with PBS alone at room temperature. They were then permeabilized in PBS containing 0.2% Triton X-100 and washed with PBS before blocking by incubation with 5% normal goat serum in PBS containing 0.05% Tween-20 for 16 h at 4°C. For interaction with primary antibodies, cells were incubated with 2.5% normal goat serum in PBS/0.05% Tween-20 containing StarD5 antibody (dilution, 1:500) for 45 min at 37°C in an incubator. For Golgi staining, GM130 antibody was used at a dilution of 1:100 together with StarD5 antibody. Macrophages were washed in PBS/0.05% Tween-20 (3 × 20 min), and the bound primary antibodies were visualized with Alexa Fluor 568 goat anti-rabbit IgG (for StarD5) or Alexa Fluor 488 goat anti-mouse IgG (for GM130). DNA was stained as described above for liver sections. After washing, coverslips containing macrophages were mounted on slides and

viewed as described above for liver sections. Control experiments were performed in the absence of the primary antibodies or with the StarD5 preimmune serum.

Dispersal of Golgi apparatus in nocodazole-treated THP-1 macrophages

THP-1 monocytes were first differentiated to macrophages on coverslips as described above. Nocodazole was dissolved in DMSO and added to the culture medium to a final concentration of 20 μM for 2 h at 37°C. Immunofluorescence detection of StarD5 protein and GM130 for Golgi staining was performed as described above.

Fluorescence labeling of THP-1 macrophages with Alexa Fluor 488 transferrin

THP-1 monocytes were first differentiated to macrophages as described above. Macrophages on coverslips were labeled with 30 μg/ml Alexa Fluor 488 transferrin for 1 h at 37°C, washed with PBS and fixed with PBS/3.7% formaldehyde for 10 min at 4°C, and then washed with PBS before blocking by incubation with 5% normal goat serum in PBS containing 0.05% Tween-20 for 1 h at room temperature in the dark. Interaction with primary antibodies (against StarD5 and GM130) was performed as described for THP-1 macrophages, and the bound primary antibodies were visualized with Alexa Fluor 568 goat anti-rabbit IgG (for StarD5) or Alexa Fluor 568 goat anti-mouse IgG (for GM130). After washing, coverslips containing macrophages were mounted on slides and viewed as described above for liver sections.

Filipin staining of cholesterol in THP-1 macrophages

After immunofluorescence staining for StarD5 and Golgi localization (as described above), THP-1 macrophages were stained with 5 mg/ml filipin in PBS plus 0.5% BSA for 30 min at 37°C and washed three times, 5 min each, with PBS while rocking gently at room temperature in the dark. The coverslips containing the cells were taken from the wells and mounted onto glass slides. The cells were allowed to dry for at least 45 min before being placed on a confocal microscope at excitation filter 360/40 nm, emission filter 460/50 nm, and beam splitter 400 nm.

Preparation of membrane fractions from THP-1 macrophages

THP-1 macrophage fractions were obtained as described previously by Li et al. (19). Briefly, cells were detached by incubating with a trypsin-EDTA solution for 1 min at 37°C. The cells were pelleted by centrifugation at 500 g for 5 min, washed with PBS, recentrifuged, resuspended into 2 ml of a low ionic strength buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride), and incubated on ice for 15 min. The cells were then homogenized in a Dounce homogenizer. Four hundred microliters of the low ionic strength buffer containing 1.46 M sucrose was added to the homogenates and centrifuged at 10,000 g for 15 min at 4°C. The pellet was stored, and the supernatant (2.4 ml) was loaded onto a sucrose density gradient tube (3.0 ml of 1.1 M sucrose, 2.6 ml of 0.88 M sucrose, and 2.6 ml of 0.58 M sucrose) and centrifuged at 100,000 g for 2 h at 4°C. This final procedure resulted in visible bands at each of the four interfaces plus a pellet, in which fraction 1 corresponds to the cytosolic fraction and fraction 2 corresponds to the Golgi.

Preparation of microsomes

THP-1 macrophage microsome fractions were obtained as described previously (17).

Overexpression and immunofluorescence detection of StarD5 in primary human hepatocytes

Human hepatocytes were plated at 15–20% of normal density in Williams E medium containing dexamethasome (0.1 μM) on six-well culture plates containing coverslips and incubated at 37°C and 5% CO_2. Twenty-four hours after plating, the cells were infected with recombinant adenovirus encoding StarD5. Two hours after infection, medium was removed and replaced with fresh medium. After 24 h, cells were immunostained with StarD5 polyclonal antibody according to the protocol described above for THP-1 macrophages. Control experiments were performed in the absence of the primary antibodies or with the StarD5 preimmune serum.

RESULTS

StarD5 is localized within Kupffer cells in the liver

Western blot analysis of human liver fractionations have previously shown StarD5 to be cytosolic, as predicted based on its lack of any targeting sequence. Surprisingly, Western blot analysis in primary human hepatocytes and endothelial cell lines (HUVE) failed to detect StarD5 protein (**Fig. 1A**). Additional Western blot analysis with up to
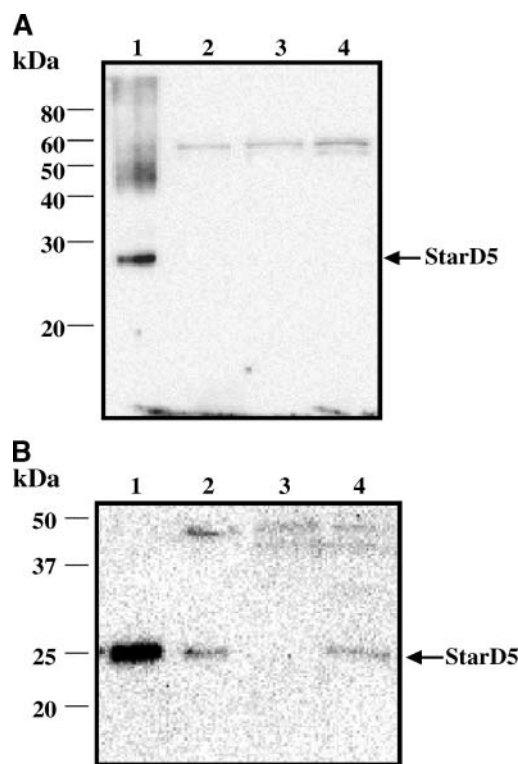

Fig. 1. StarD5 is expressed in nonparenchymal cells in the liver. A: Western blot analysis with StarD5 polyclonal antibody failed to detect StarD5 protein in primary human hepatocytes (10 μg) (lane 2), HepG2 cells (10 μg) (lane 3), and human endothelial cells (HUVE; 10 μg) (lane 4). B: Western blot analysis with StarD5 polyclonal antibody confirmed the presence of StarD5 protein in whole human liver extracts (60 μg) (lane 2) as well as its presence in nonparenchymal cells from human liver (60 μg) (lane 4), but it was not detected in freshly isolated human hepatocytes (60 μg) (lane 3). Lanes 1 in both blots show StarD5 recombinant protein.

100 µg of protein from primary human hepatocyte cultures was performed without detection of StarD5 protein (data not shown). Addressing the possibility of hepatocyte dedifferentiation in culture and their loss of StarD5 expression, cells from freshly isolated human hepatocyte suspensions were also analyzed (Fig. 1B). Western blot analysis failed to detect StarD5 protein in these purified hepatocyte cell suspensions (hepatocyte purity of >98%). On the other hand, the same analysis showed the presence of StarD5 protein in nonparenchymal cells (Fig. 1B). To further localize this protein to specific cells within the liver, immunolocalization studies on human liver sections
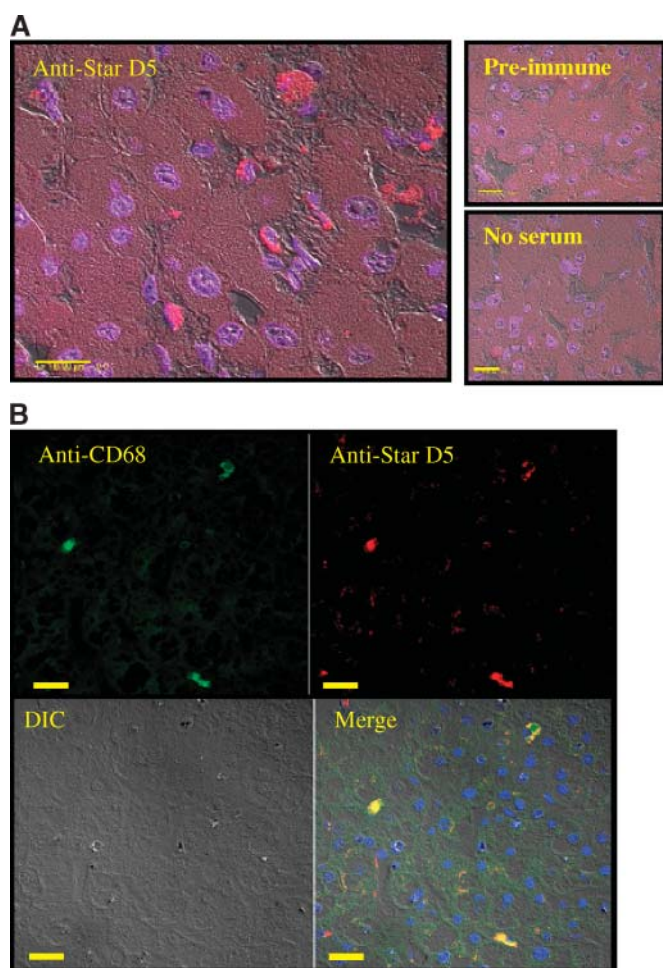
were carried out. **Figure 2** shows the presence of StarD5 in cells located next to sinusoids (Fig. 2A). This observation, coupled with the fact that two other major cell types in the liver (hepatocytes and endothelial cells; Fig. 1) do not appear to express StarD5, led us to hypothesize that it might be expressed in Kupffer cells. To confirm this hypothesis, double immunofluorescence confocal microscopy studies were performed on normal human liver sections with polyclonal StarD5 antibody and with the murine antibody to CD68, a marker for Kupffer cells. Immunofluorescence analysis of the human liver sections confirmed the colocalization of StarD5 and CD68 in cells lining the hepatic sinusoids, corresponding to Kupffer cells (Fig. 2B). Immunoreactivity was absent in liver sections without the primary antibody or with the StarD5 preimmune serum (Fig. 2A).

StarD5 is expressed in immune/inflammatory-related cells

The presence of StarD5 in Kupffer cells, the liver macrophage equivalents, led us to look for StarD5 in monocytes and macrophages. Western blot analysis
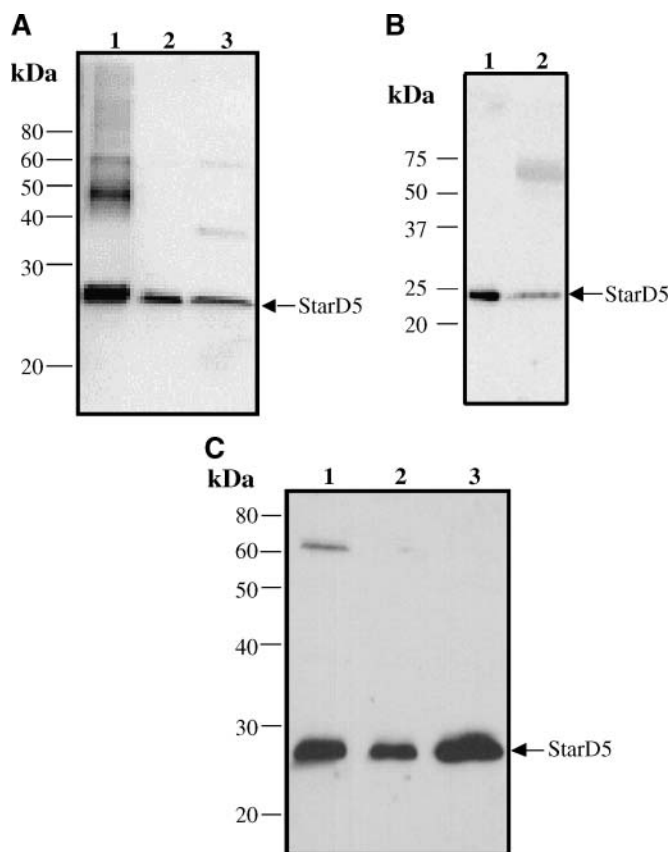

Fig. 2. StarD5 is expressed in Kupffer cells within the liver. A: 4′,6-Diamidino-2-phenylindole (DAPI; nuclear staining) and anti-StarD5 immunofluorescence confocal microscopy analysis in human liver sections. The left panel shows the localization of StarD5 within the liver section. StarD5 polyclonal antibody is indicated by red stain; nuclear staining is blue. The right panels show two negative controls for StarD5 using preimmune StarD5 serum (top) or without serum (bottom). Bars = 18 µm. B: DAPI (nuclear staining), anti-StarD5, and anti-CD68 double immunofluorescence microscopy analysis of a human liver section. The top left panel shows the localization of Kupffer cells with the CD68 antibody (green). The top right panel shows the localization of StarD5 with the StarD5 polyclonal antibody (red). The bottom left panel shows a differential interference contrast (DIC) image of the liver section. The bottom right panel shows the colocalization of StarD5 and CD68 in Kupffer cells (yellow). Bars = 20.2 µm. Original magnification, ×63.

Fig. 3. StarD5 is expressed in THP-1 monocytes/macrophages, primary human monocytes, and other immune-related cells. Western blot analysis was carried out with StarD5 polyclonal antibody. A: Lane 1, StarD5, recombinant protein (150 ng); lane 2, THP-1 monocytes (10 µg); lane 3, THP-1 differentiated macrophages (10 µg). B: Lane 1, StarD5, recombinant protein (100 ng); lane 2, primary human monocytes (10 µg). C: Lane 1, HMC-1 human mast cells (10 µg); lane 2, KU 812-F human lymphoblasts (10 µg); lane 3, HL-60 human promyeloblasts (10 µg).

performed on THP-1 monocytes and THP-1 differentiated macrophages with the StarD5 polyclonal antibody showed the expression of StarD5 protein in both cell types (**Fig. 3A**). To establish the relevance of THP-1 monocytes and macrophages to the human, primary human monocytes were isolated from a human blood sample according to the protocol described in Materials and Methods. StarD5 protein was detected in primary human monocytes after Western blot analysis using the StarD5 polyclonal antibody. The fact that StarD5 was detected only in immune-related cells (Kupffer cells, monocytes, and macrophages) led us to analyze other available immune-related cells. Western blot analysis with the StarD5 polyclonal antibody performed with three different cell lines, promyeloblasts (HL-60), mast cells (HMC-1), and lymphoblasts (KU 812-F), showed the presence of the protein in these three cell types (Fig. 3B). Further Western blot analysis on readily available tissues and cell lines (brain tissue, fibroblasts, osteosarcoma, and astrocytes) did not detect the presence StarD5 (data not shown).

StarD5 is localized in cytosol and the Golgi

We have shown previously (17) that StarD5 is localized in the cytosol by subcellular fractionation of liver tissue, but the high levels of StarD5 protein observed in THP-1 monocytes/macrophages (Fig. 3A) made these cells more suitable for immunolocalization analysis. Immunofluorescence studies with the StarD5 antibody showed the protein widely distributed in the cytosol of THP-1 macrophages, but with a focal intensity in an area localized next to the cell nucleus (**Fig. 4A**). Based on the high concentration of the protein in a perinuclear area and the association of increased StarD5 expression with increased cholesterol within the microsomal fraction (17), we hypothesized that StarD5 could localize to the Golgi. To confirm this, we performed double immunofluorescence studies using a mouse IgG against GM130 (a Golgi marker) together with the StarD5 antibody. Figure 4C shows immunofluorescence staining of StarD5 in THP-1 macrophages, which corroborates the presence of the protein not only in the cytosol (Fig. 4A) but in close association with a perinuclear organelle, the Golgi (Fig. 4C). Immunoreactivity was absent in THP-1 macrophages when primary antibody was not added or with the StarD5 preimmune serum (Fig. 4B).

THP-1 macrophage cell fractions were then examined by Western blot analysis to determine StarD5 localization. StarD5 protein was detected in the cytosolic fraction (**Fig. 5A**) but not in the nuclear, Golgi, plasma membrane, or ER fraction (Fig. 5A, lanes 1, 3, 4, and 6). Microsomal preparation showed StarD5 to be localized to the cytosolic fraction (Fig. 5B).
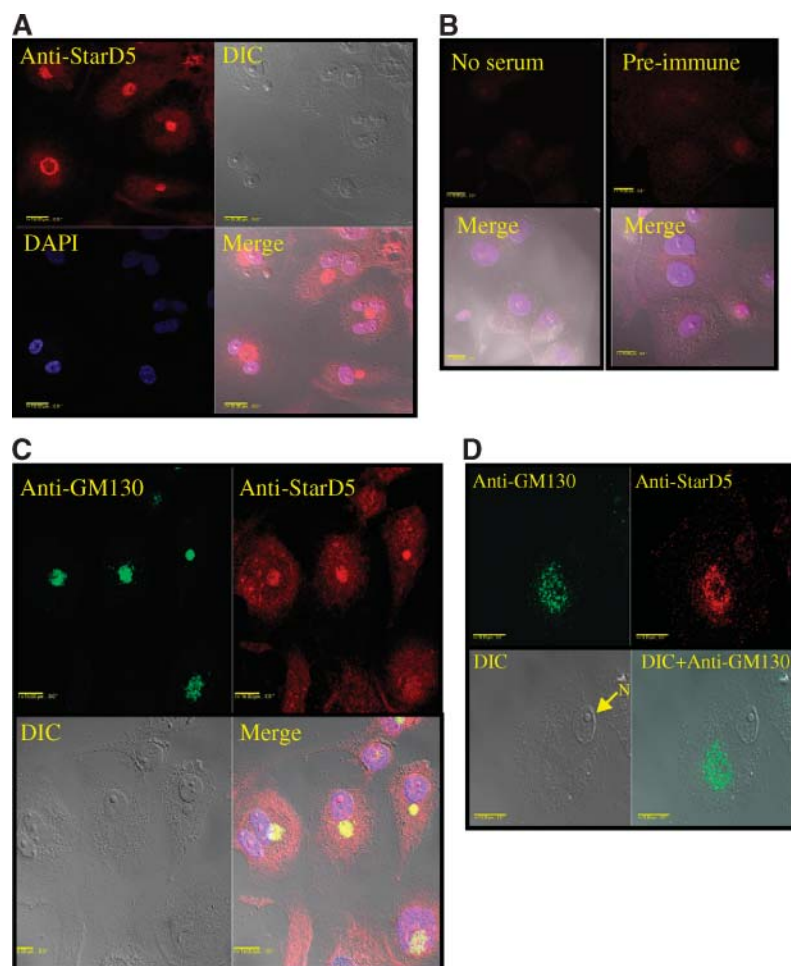

Fig. 4. StarD5 is localized in the cytosol and the Golgi in macrophages. A: DAPI (nuclear staining) and anti-StarD5 immunofluorescence confocal microscopy analysis in THP-1 macrophages. The top left panel shows the localization of StarD5 with the StarD5 polyclonal antibody (red); the top right panel shows a differential interference contrast (DIC) image; the bottom left panel shows nuclear staining (blue; of note is that THP-1 cells are frequently found to be multinucleate); the bottom right panel shows the merged image obtained by superimposing the three images mentioned above. B: The panels show two negative controls for StarD5, without serum (left panels) or using preimmune StarD5 (right panels). C: DAPI (nuclear staining), anti-StarD5, and anti-GM130 double immunofluorescence microscopy analysis of THP-1 macrophages. The top left panel shows Golgi staining with the GM130 antibody (green); the bottom left panel shows a DIC image of the macrophages; the top right panel shows the localization of StarD5 (red); the bottom right panel shows the colocalization of StarD5 and GM130 in the Golgi of the cells. Bars = 16 μm. D: Dispersal of the Golgi apparatus and StarD5 protein in nocodazole-treated THP-1 macrophages. The top left panel shows Golgi staining with the GM130 antibody (green); dispersion of the Golgi can be appreciated by its speckled appearance. The top right panel shows the localization of StarD5 (red); the bottom left panel shows a DIC image (N, nucleus); the bottom right panel shows the merged image obtained by superimposing the DIC image and the Golgi staining. Bars = 20 μm. Original magnification, ×63.
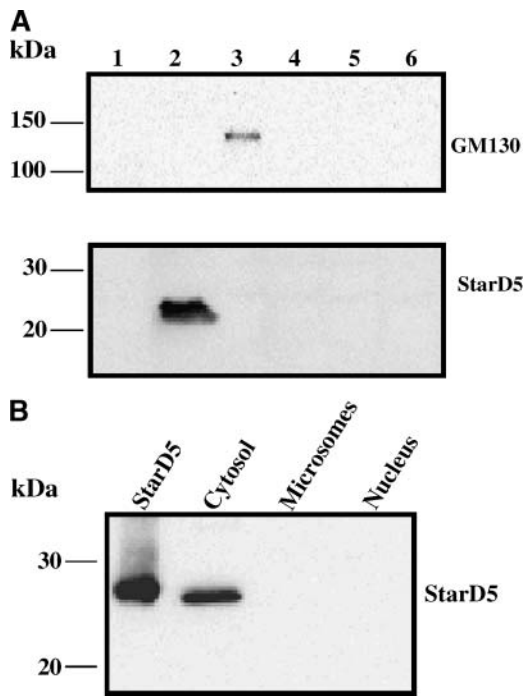
Fig. 5. StarD5 is localized in the cytosol after cell fractionation. A: Using gradient sucrose fractionation (see Materials and Methods), Western blot analysis with StarD5 polyclonal antibody and the Golgi marker GM130 was carried out on the different subcellular fractions (15 μg of protein) isolated from THP-1 macrophages. Lane 1, initial pellet; lane 2, fraction 1; lane 3, fraction 2; lane 4, fraction 3; lane 5, fraction 4; lane 6, final pellet. StarD5 protein was detected only in the cytosolic fraction of THP-1 macrophages. B: Western blot analysis with StarD5 polyclonal antibody was also carried out on microsomal preparations (i.e., in fractions containing both ER and Golgi fractions) obtained via ultracentrifugation (see Materials and Methods) from THP-1 macrophages. StarD5, StarD5 pure protein (200 ng); Cytosol, cytosolic fraction (25 μg); Microsomes, microsomal fraction (25 μg); Nucleus, nuclear fraction (25 μg). StarD5 protein was again detected only in the cytosolic fraction of THP-1 macrophages.

To determine whether StarD5 is associated with the Golgi and not with the perinuclear endocytic recycling compartment (ERC), the Golgi apparatus was dispersed in nocodazole-treated THP-1 macrophages. The addition of nocodazole to the culture medium led to the fragmentation and dispersion of Golgi membranes. StarD5 also underwent dispersion in a pattern consistent with that observed for the Golgi (Fig. 4D). Also, using an ERC marker (Alexa Fluor 488 transferrin), we were able to identify the ERC in THP-1 macrophages as having a perinuclear localization similar to that described for StarD5 and the Golgi. However, the ERC stained more diffusely over a greater area than was observed for StarD5 staining, which exhibited a focal intensity similar to Golgi morphology. It was also observed that the Golgi and StarD5 were at the same focal plane, whereas the ERC was in a different focal plane (data not shown). Furthermore, the cytosolic localization (staining pattern) of StarD5 differed from that of endocytic Alexa Fluor 488 transferrin.

Recent findings have suggested the presence of StarD5 in the nucleus, observations that we have been unable to confirm in monocyte/macrophage studies. To further explore the possible presence of StarD5 in the nucleus, primary human hepatocytes were infected with a recombinant StarD5 adenovirus. After overexpression of StarD5, anti-StarD5 immunofluorescence showed StarD5 in the expected diffuse localization throughout the cytoplasm of the cells. No nuclear staining of StarD5 was found (data not shown).

We reported previously that StarD5 overexpression in primary rat hepatocytes led to an increase of free cholesterol in the microsomes (17). Based upon this result and the immunocytochemistry studies shown above, we hypothesized that increasing amounts of free cholesterol in THP-1 macrophages are localized in association with the Golgi. To confirm this hypothesis, cellular free cholesterol was stained with filipin in THP-1 macrophages previously immunostained for StarD5 and Golgi localization. As shown above, StarD5 was located again in the cytosol, with a concomitant increase of red staining with the Golgi (green) (**Fig. 6A**, anti-GM130 and anti-StarD5 panels). Filipin staining was diffuse in the cytosol and membranes, but with an area of highly increased fluorescence, indicating the presence of high levels of free cholesterol (Fig. 6A, filipin panel). Figure 6B shows extensive colocalization of StarD5 and Golgi (left panel), free cholesterol and Golgi (center panel), and free cholesterol and StarD5 (right panel). These results support the hypothesis that THP-1 macrophages accumulate free cholesterol in/ next to the Golgi and that StarD5 might play a major role in the distribution of free cholesterol in the cell.

DISCUSSION

Recent studies on the different proteins of the StarD4 subfamily have reported the first data on localization, regulation, and binding specificities of StarD4, StarD5, and StarD6 (14, 15). Although StarD5 seems to be widely expressed, with the liver showing the highest levels of StarD5 mRNA (2, 12), we have previously shown that protein levels in the liver are low (17). Immunolocalization of StarD5 protein in human liver sections confirmed previous findings of detection within the liver (17), but only in hepatic sinusoidal lining cells (Fig. 2A). A double immunofluorescence analysis showed that those sinusoidal lining cells also reacted with a monoclonal antibody to CD68 (Fig. 2B), a specific marker for monocytes/ macrophages and Kupffer cells, demonstrating that StarD5 protein is expressed within Kupffer cells in the liver. Additional analysis showed that no primary human hepatocytes, freshly isolated hepatocytes, or HepG2 cells contained detectable StarD5 protein (Fig. 1). Western blot analysis of another common cell type in the liver, the endothelial cell, also failed to detect StarD5 protein (Fig. 1).

Not surprisingly, given the expression in Kupffer cells, Western blot analysis with the StarD5 polyclonal antibody showed expression of the protein in THP-1 monocytes and
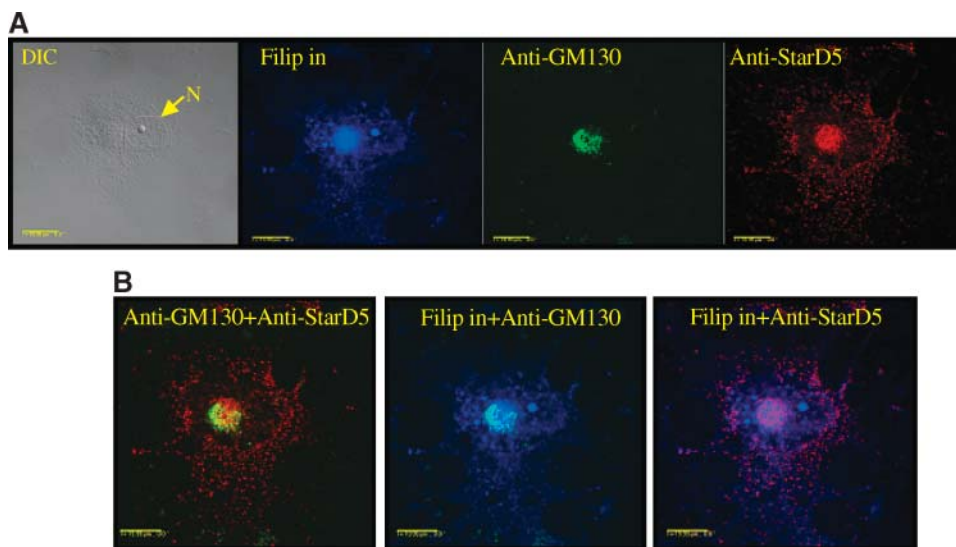
Fig. 6. StarD5 is associated with high levels of free cholesterol. A: From left to right, panels show a differential interference contrast (DIC) image (N, nucleus), filipin staining of free cholesterol (blue), staining of Golgi with anti-GM130 (green), and StarD5 staining with anti-StarD5 (red) of a THP-1 macrophage. B: Merged images of THP-1 macrophages stained with filipin, anti-StarD5, and anti-GM130. From left to right, panels show merged images of anti-GM130 + anti-StarD5, filipin + anti-GM130, and filipin + anti-StarD5, intended to demonstrate the extent of colocalization among the three stainings/markers. Bars = 20 μm. Original magnification, ×63.

macrophages and primary human monocytes (Fig. 3A). Interestingly, promyelocytic cells, as well as other, more differentiated inflammatory cell lines (mast cells and basophils), also demonstrated very high StarD5 protein levels (Fig. 3B).

We reasoned that StarD5's immunolocalization in macrophages might provide a clue to the role of the protein in cellular cholesterol homeostasis and the mechanism of cholesterol transport. Figure 4A confirms the localization of StarD5 in the cytosol of the cells, but, interestingly, the protein seems to be most highly concentrated in an area next to the nucleus of the cells. Further double immunofluorescence studies with StarD5 polyclonal antibody and GM130 antibody (a Golgi marker) confirmed that StarD5 was not only in the cytosol but appears to be intensely associated with the Golgi (Fig. 4B). Further studies to confirm the localization of StarD5 with the Golgi showed that fragmentation and dispersion of the Golgi by disruption of the microtubule structure with nocodazole (20) also causes fragmentation and dispersal of the membranes in which StarD5 is located in the perinuclear region (Fig. 4D). Our data also indicate that the disruption of the microtubule structure causes a similar pattern of dispersal of Golgi membranes and the membranes in which StarD5 is localized, which supports the localization of StarD5 with the Golgi in THP-1 macrophages. When Alexa Fluor 488 transferrin was used for the staining of the ERC, its morphology did not resemble the highly intense perinuclear staining of StarD5. Of additional note is that the endocyte-localized fluorescent transferrin did not colocalize with the cytosolic StarD5. Although these findings are supportive of a role of StarD5

in the nonvesicular movement of cholesterol, it is not yet possible to exclude a possible role for StarD5 in the vesicular movement of cholesterol. Furthermore, although unlikely, the artifactual fixing and staining of soluble StarD5 with Golgi should also be considered. In summary, the combined gradient and immunofluorescence data support a possible mechanism for cholesterol transport between cell membranes and the Golgi similar to that described for other START proteins predicted to be cytosolic (12, 13, 16), with a loose association of StarD5 with the Golgi membrane.

The high levels of free cholesterol and extensive colocalization with the Golgi membranes and StarD5 (Fig. 6B) observed in THP-1 macrophage cells suggest that this organelle accumulates free cholesterol in association with StarD5. The labeling of THP-1 macrophages with a fluorescent human transferrin showed evidence of an ERC, a major sterol storage compartment in mammalian cells that is closely related to the *trans*-Golgi network (21–23). These observations led us to assign the densest localization of free cholesterol to the Golgi in THP-1 macrophages. Coupled with the cytosolic localization of StarD5 in subcellular fractions (Fig. 5) and the ability to transfer cholesterol to the microsomes (17), these findings support a mechanism for cholesterol transport to the Golgi, an ability to transport cholesterol like other START proteins predicted to be cytosolic (12, 13, 16). In contrast to the studies by Alpy and Tomasetto (16), immunolocalization studies of StarD5 did not show any evidence of nuclear localization in either macrophages (Fig. 4A) or primary human hepatocytes (data not shown) infected with a recombinant adenovirus encoding StarD5.

The high expression of StarD5 in monocytes/macrophages, and its mRNA induction in free cholesterol-loaded macrophages (15), raises many questions about its possible role in cholesterol homeostasis. Just as interestingly, StarD5 selectively binds 25-hydroxycholesterol, a potent regulatory metabolite of cholesterol. Given the recent observations of this and earlier studies, it is plausible that StarD5 may play a protective role in ER-stressed macrophages. StarD5, which is expressed in macrophages under normal conditions, would be upregulated under ER stress conditions caused by high levels of ER cholesterol (or 25-hydroxycholesterol). It might then transport free cholesterol/oxysterol from the ER membranes to the Golgi and thus reduce the accumulation of free cholesterol/oxysterol in the ER. This mechanism would have important implications for atherogenic plaques in macrophages/foam cells, possibly aiding in the cell's survival as a response to the ER stress caused by excess ER cholesterol.⬛

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