Identification of Megalin/gp330 as a Receptor for Lipoprotein(a) In Vitro

Andreas Niemeier, Thomas Willnow, Hans Dieplinger, Christian Jacobsen, Nicolette Meyer, Jan Hilpert, Ulrike Beisiegel

Abstract—Lipoprotein(a) [Lp(a)] is an atherogenic lipoprotein of unknown physiological function. The mechanism of Lp(a) atherogenicity as well as its catabolic pathways are only incompletely understood at present. In this report, we show that the low density lipoprotein receptor (LDLR) gene family member megalin/glycoprotein (gp) 330 is capable of binding and mediating the cellular uptake and degradation of Lp(a) in vitro. A mouse embryonic yolk sac cell line with native expression of megalin/gp330 but genetically deficient in LDLR-related protein (LRP) and a control cell line carrying a double knockout for both LRP and megalin/gp330 were compared with regard to their ability to bind, internalize, and degrade diocadecyltetramethylindocarbocyanine perchlorate (DiI)-fluorescence–labeled Lp(a) as well as equimolar amounts of 125I-labeled Lp(a) and LDL. Uptake and degradation of radiolabeled Lp(a) by the megalin/gp330-expressing cells were, on average, 2-fold higher than that of control cells. This difference could be completely abolished by addition of the receptor-associated protein, an inhibitor of ligand binding to megalin/gp330. Mutual suppression of the uptake of 125I-Lp(a) and of 125I-LDL by both unlabeled Lp(a) and LDL suggested that Lp(a) uptake is mediated at least partially by apolipoprotein B100. Binding and uptake of DiI-Lp(a) resulted in strong signals on megalin/gp330-expressing cells versus background only on control cells. In addition, we show that purified megalin/gp330, immobilized on a sensor chip, directly binds Lp(a) in a Ca2+-dependent manner with an affinity similar to that for LDL. We conclude that megalin/gp330 binds Lp(a) in vitro and is capable of mediating its cellular uptake and degradation. (Arterioscler Thromb Vasc Biol. 1999;19:552-561.)

Key Words: megalin ■ glycoprotein 330 ■ lipoprotein(a) ■ LDL ■ LDLR gene family

Lipoprotein(a) [Lp(a)] is a lipoprotein particle in human plasma with close structural relationship to LDL. Complexed with lipids, the major structural protein component of both LDL and Lp(a) is apolipoprotein (apo) B100. Lp(a) differs from LDL in that the former contains apo(a) as an additional apolipoprotein that is covalently linked to apoB100 (for a review, see References 1 and 2). Apo(a) is a highly polymorphic glycoprotein that shares sequence homology with plasminogen, owing to the presence of cysteine-rich protein motifs, the so-called “kringles,” and an inactive protease domain that is common to several serine proteases of protein motifs, the so-called “kringles,” and an inactive protease domain that is common to several serine proteases of
the LDLR and the LDLR-related protein (LRP) in vitro.\textsuperscript{25} Taken together, the prevailing view today is that the LDLR is capable of binding Lp(a) with low affinity but most likely does not represent a catabolic pathway of major importance in vivo.

Of all known LDLR gene family members, the VLDL receptor (VLDLR) is closest in structure to that of LDLR. On the basis of adenovirus-mediated LDLR and VLDLR over-expression in fibroblasts, Lp(a) has recently been shown to bind not only to the LDLR but also to the VLDLR.\textsuperscript{26} Megalin/glycoprotein 330 (gp330) was first identified as the major autoantigen in Heymann’s nephritis, a rat model for membranous glomerulonephritis.\textsuperscript{27,28} Subsequent structural and functional work led to its classification into the LDLR gene family\textsuperscript{29–32} and to the characterization of a multitude of heterogeneous ligands for this 600-kDa endocytotic receptor. Ligands include elements of lipoprotein metabolism, of the blood clotting and fibrinolytic systems, calcium, polybasic drugs, and others,\textsuperscript{33–44} including a receptor-associated protein (RAP).\textsuperscript{35} RAP associates intracellularly with megalin/gp330.
Degradation of 125I-tPA, 125I-uPA, and 125I-GST-RAP by yolk sac cells. Replicate monolayers of cell lines 10A, 1461, and 6A3 received 1 mL DMEM (without glutamine) containing 0.2% (wt/vol) BSA and either 1 mg/mL GST-RAP (A; specific activity, 1215 cpm/ng), 100 ng/mL 125I-uPA/PPACK (B; specific activity, 3800 cpm/ng), or 100 ng/mL 125I-tPA/YPACK (C; specific activity, 86 850 cpm/ng). After incubation at 37°C for the indicated periods of time, the amount of 125I-labeled degradation products secreted into the medium was determined. Each value represents the mean of duplicate incubations.

**Methods**

**Generation of Yolk Sac Cell Lines**

To generate cell lines 10A and 1461, individual embryos from matings of mice heterozygous for the LRP gene defect (LRP+/−) were isolated at day 10.5 of gestation, and the embryonic membranes were removed. The yolk sacs were placed in ice-cold, 0.05% trypsin-EDTA solution and kept overnight at 4°C. On the following day, the samples were incubated at 37°C and disaggregated by vigorous pipetting. The cell suspension was plated on a 60-mm culture dish and grown to confluence. Individual cell clones were genotyped by Southern blot analysis. To immortalize all yolk sac lines, they were transfected with a plasmid encoding simian virus 40 large T antigen.

**Cell Culture and Protein Preparation**

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) without glutamine and with 10% FCS. For protein blotting procedures, cells were solubilized in 1% Triton X-100, 50 mmol/L Tris, 2 mmol/L CaCl2, and 80 mmol/L NaCl, pH 8.0. A mix of protease inhibitors (Calbiochem) was added, including 1 mmol/L pepstatin A, 10 mmol/L chymostatin, 10 mmol/L leupeptin, and 10 mmol/L antipain. Protein concentrations were determined by standard procedures according to Lowry et al.

**Gel Electrophoresis and Antibodies**

SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to Neville. For 125I-labeled proteins, gels were dried and exposed to an autoradiography film (Cronex, DuPont). Nonlabeled proteins were electroblotted to nitrocellulose for nonspecific protein stain (Ponceau S solution, Serva) and immunodetection with specific antibodies. Polyclonal antibodies against megalin/gp330, LRP, and VLDLR were given to us by J. Herz (University of Texas Southwestern Medical Center, Dallas, Tex), and the polyclonal anti-VLDLR was kindly provided by M. Gåfvels (Karolinska Institute, Huddinge, Sweden). Peroxidase-labeled goat anti-rabbit antibodies (Dianova) were used with chloronaphthol as the substrate for secondary antibodies. Southern blot analyses were performed according to standard procedures.

**Lipoprotein Purification**

Lp(a) was purified from 500 mL of fresh EDTA-plasma of a healthy proband undergoing plasmapheresis by sequential 3-step ultracentrifugation as described previously. The Lp(a) concentration was 52 mg/dL, with an apo(a) isoform of 21, as determined by SDS agarose gel electrophoresis. Other isoforms from additional donors (S1, S2, and S3, according to the former nomenclature) were isolated by the same method. LDL was obtained from the same donor as described before.

**Lipoprotein Characterization and Labeling**

Lipoproteins were separated on an agarose gel ready kit (Sebia GmbH) and stained with Sudan black solution. Each Lp(a) and LDL
preparation was subjected to 5% to 7.5% SDS-PAGE under reducing conditions, followed by electroblotting to nitrocellulose, and subsequent Ponceau staining or immunoblotting with an anti-apo(a) and/or apoB100 antibody \(^5\) (data not shown); only pure preparations were used in the cell assays. For iodination the ICl method was used. \(^5\) Typically, the specific activity of \(^{125}\)I-Lp(a) was within the range 150 to 300 counts per minute (cpm)/ng, and that of \(^{125}\)I-LDL, between 50 and 150 cpm/ng. After iodination, \(^{125}\)I-labeled lipoproteins were checked again for integrity by SDS-PAGE.

Cellular Uptake and Degradation of \(^{125}\)I-Labeled Ligands: uPA, tPA, and RAP

Complexes of \(^{125}\)I-urokinase-type plasminogen activator (uPA)/PPACK and \(^{125}\)I-tissue plasminogen activator (tPA)/YPACK were prepared as described. \(^5\) PPACK (Phe-Pro-Arg chloromethyl ketone) and YPACK (Tyr-Pro-Arg chloromethyl ketone) are inhibitors of uPA and tPA catalytic activity, respectively, and are needed to prevent uPA and tPA from exerting their protease activity in the cellular assays. All proteins were radiolabeled by the Iodo-Gen (Pierce) method. \(^5\) Yolk sac cells (2×10\(^5\) per well) were seeded into 12-well plates and grown for 24 hours. The medium was replaced with DMEM (without glutamine) containing 0.2% (wt/vol) BSA and the indicated iodinated ligands. Cellular degradation of \(^{125}\)I-labeled proteins was measured as previously described \(^5\) and is expressed as nanograms of \(^{125}\)I-labeled trichloroacetic acid–soluble (noniodide)
material released into the culture medium per milligram of total cell protein.

Uptake and Degradation of 125I-Labeled Lipoproteins

Cells (5 × 10^4/mL) were seeded routinely on day 0 in 24-well plates and used as confluent monolayers on day 2. The experiments were performed in DMEM containing 5% BSA (fraction V, Sigma) and 0.02 mol/L HEPES (pH 7.4). Aliquots (5 to 70 picomoles of 125I-Lp(a) and 125I-LDL per milliliter) were added. All data points were obtained in duplicate. For the differentiation between total, specific, and nonspecific uptake and degradation, a 12- to 50-fold molar excess of unlabeled lipoprotein was added. RAP in the form of a recombinant polyhistidine fusion peptide was kindly provided by J. Gliemann, Aarhus, Denmark, and was added at a concentration of 30 μg/mL. For the determination of cellular uptake, incubations were performed for 90 minutes at 37°C. After incubation, cells were washed with PBS, pH 7.4, with 2 mg/mL BSA, followed by a short PBS rinse without BSA. Surface-bound lipoproteins were then released by PBS containing 770 U heparin per milliliter (Liquemin, Roche). The cells were dissolved in 0.1 mol/L NaOH. Finally, radioactivity and cell protein of the lysate were determined. Specific uptake data are expressed as nanograms or femtomoles of ligand protein per milligram of cell protein. Degradation was measured after incubation for 90 minutes followed by a 4-hour chase. After the chase, the media were recovered completely, and 125I-labeled trichloroacetic acid–soluble material was determined as a direct measure of degraded 125I-apolipoproteins.

Immunofluorescence

For immunofluorescence studies, Lp(a) was labeled with 1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) according to standard procedures. The integrity of labeled Lp(a) was checked by agarose gel electrophoresis. Cells were grown on glass coverslips for 2 days, washed, and incubated with 100 pmol/mL DiI-labeled Lp(a) at 37°C for 20 minutes in DMEM containing 1% BSA. Subsequently, coverslips were washed, and surface-bound Lp(a) was released by 500 U/mL heparin (Sigma) at 4°C for 15 minutes. Cells were fixed at room temperature in 4% paraformaldehyde and permeabilized with methanol at −20°C for 5 minutes. After extensive washing, blocking was performed at room temperature with 1% BSA, 10% goat serum, and 20 mmol/L glycine in PBS. Cells were incubated with a polyclonal antibody against megalin/gp330 from rabbit at 37°C for 60 minutes, washed twice, and incubated at 37°C for 45 minutes with DTAI 5-(4,6-dichlorotriazin-2-yl)amino)fluorescein–conjugated goat anti-rabbit immunoglobulins from Dianova. Coverslips were mounted on a glass slide with a drop of Mowiol (Calbiochem). Confocal laser scanning microscopy was performed using a Leica TCS 4D (Leica Lasertechnik) instrument based on an inverted Leitz DMIRBE microscope interfaced with an Ar-Kr laser adjusted to 488 and 568 nm.

Biosensor Measurements

All measurements were performed on a BIAcore 2000 instrument (Biosensor) equipped with CM5 sensor chips maintained at 20°C. A continuous flow of HBS buffer (10 mmol/L HEPES, pH 7.4; 3.4 mmol/L EDTA; 0.15 mol/L NaCl; and 0.005% surfactant P20) passing over the sensor surface was maintained at 5 μL/min. The carboxylated dextran matrix of the sensor chip was activated by injection of 60 μL of a solution containing 0.2 mol/L N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and 0.05 mol/L N-hydroxysuccinimide in water. LDLR was injected at a concentration of 40 μg/mL in 10 mmol/L sodium acetate, pH 4.0 (total volume of 300 μL per flow cells 1 and 2). Megalin was at a concentration of 10 μg/mL in 10 mmol/L sodium acetate, pH 4.5 (total volume of 350

Figure 5. Uptake of DiI-Lp(a) by yolk sac cells. 1461 (A through C) and 6A3 (D through F) cells were incubated with DiI-fluorescence–labeled Lp(a) (red) at a concentration of 100 pmol/mL, and uptake was allowed to proceed for 20 minutes at 37°C. Immunodetection of megalin/gp330 (A, C, D, and F) was performed with a polyclonal antibody, followed by an DTAI-conjugated secondary goat anti-rabbit antibody (green). Panels C and F result from the superimposition of DiI-Lp(a) (B and E) and megalin/gp330 fluorescence (A and D). Experimental details and laser scanning microscopy are described in Methods. Bar = 10 μm.
The remaining binding sites were blocked by injection of 35 μL of 1 mol/L ethanolamine, pH 8.5. The immobilized protein in flow cells 2 and 4 was then reduced by injection of 100 μL of 0.5% DTT in 6 mol/L guanidine HCl, 5 mmol/L EDTA, and 50 mmol/L Tris, pH 8.0, into each flow cell. The surface plasmon resonance signal from immobilized LDLR generated 3107 BIAcore response units (RUs), equivalent to 19 fmol LDLR per mm², and immobilized megalin generated 10 343 BIAcore RUs, equivalent to 17 fmol megalin per mm². Screening of the LDL and Lp(a) samples was performed by injecting aliquots of 80 μL at concentrations of 10 to 100 μg/mL through all flow cells at a flow rate of 10 μL/min. The samples were dissolved in 10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1.5 mmol/L CaCl₂; 1 mmol/L EDTA; and 0.005% surfactant P20. The same buffer was used as the running buffer. The BIAcore response is expressed in relative RUs, i.e., the difference in response between the immobilized protein flow cell and the parallel reduced-protein flow cell. Regeneration of the sensor chip after each analysis cycle was performed by injecting 5 μL of 0.05% SDS. Dissociation constants were calculated from fitted curves by means of the BIAcore software program version 4.0.

Results

Generation of Cell Lines 1461 (LRP⁺⁻) and 6A3 (LRP⁻⁻, Megalin/gp330⁺⁻)

Mouse embryonic yolk sac cell lines 1461 and 6A3 were generated for functional analysis of megalin/gp330. Because many ligands recognized by megalin/gp330 can also bind to LRP, it was mandatory to rule out potential interference from the LRP. For this purpose, both alleles of the LRP gene were disrupted in cell lines 1461 and 6A3 (Figure 1A, lanes 1 through 3) while the alleles of the megalin/gp330 gene were left intact in 1461 but disrupted in the negative control cell line 6A3 only (Figure 1A, lanes 4 and 5). Western blot analysis confirmed the complete absence of LRP protein expression by both cell lines as well as exclusive expression of megalin/gp330 by 1461 cells, according to their genotype (Figure 1B, lanes 1 through 6). LDLR was expressed by cell line 1461 as well as by 6A3 cells. The amount of immuno-detectable protein without (data not shown) and after LDLR stimulation, induced by incubation with lipoprotein-deficient serum for 2 days, was slightly higher for 1461 cells (Figure 1B, lanes 7 through 9). VLDLR protein could not be detected in either cell line (Figure 1B, lanes 10 through 12).

Functional Integrity of Megalin/gp330 Expressed by Cell Line 1461

To ensure that megalin/gp330 was functionally intact in 1461 cells and absent in 6A3 cells, we compared 1461 and 6A3 cell lines with regard to their ability to degrade established ligands of megalin/gp330. Over a time course of 20 hours, GST-RAP and uPA were efficiently degraded by megalin/gp330-expressing 1461 cells but were degraded only to a very minor degree by the megalin/gp330-negative control cell line 6A3 (Figure 2A and 2B). tPA has been reported to be degraded by LRP but not by megalin/gp330.58 Irrespective of megalin/gp330 expression, no tPA degradation was observed by the LRP-negative cell lines 1461 and 6A3 (Figure 2C), whereas tPA
Megalin/gp330 Binds Lipoprotein(a)

Characterization of Purified Lp(a) and LDL

Freshly isolated human Lp(a) and LDL from a healthy human subject with the slowly migrating, large apo(a) isoform 21 were checked for purity and integrity before and after ¹²⁵I radiolabeling. Figure 3A shows the agarose gel electrophoresis pattern of the major lipoproteins from the donor. To ensure that only intact lipoprotein preparations were used, SDS-PAGE of unlabeled (Figure 3B) and radiolabeled (Figure 3C) Lp(a) and LDL was routinely performed in addition. These tests demonstrated that the preparations contained neither significant amounts of degradation products nor contamination by other proteins. In particular, to check for the presence of other receptor-binding apolipoproteins, higher-percentage gels (12% to 15%) of Lp(a) and LDL were immunoblotted against apoE and apoAI. Neither one was detectable (data not shown).

Megalin/gp330-Mediated Cellular Uptake and Degradation of ¹²⁵I-Lp(a)

We measured the cellular uptake and degradation of ¹²⁵I-Lp(a) by the cell line 1461, which expresses native amounts of megalin/gp330. When these cells were incubated with increasing amounts of ¹²⁵I-Lp(a), cellular degradation of ¹²⁵I-Lp(a) appeared to be saturable in a characteristic fashion (Figure 4A) as is observed for specific receptor-ligand interactions, such as LDL degradation via the LDLR pathway. Strikingly, specific degradation of ¹²⁵I-Lp(a) made up <50% of total degradation, leaving a substantial portion of cellular degradation whose exact nature remains unclear at the moment. We were interested in the specific and thus, presumably receptor-mediated portion of uptake, in particular, asking the question whether this specificity was due to megalin/gp330 interaction with Lp(a); therefore, we performed another set of comparative uptake and degradation assays including the megalin/gp330-deficient control cell line 6A3. These experiments were performed with the addition of RAP as a highly competent and specific inhibitor of ligand binding to megalin/gp330. Uptake (Figure 4B) and degradation (Figure 4C) of ¹²⁵I-Lp(a) were consistently higher on megalin/gp330-expressing 1461 cells compared with the control 6A3 cells. On average, uptake and degradation of ¹²⁵I-Lp(a) by the megalin/gp330-negative control cells were ≈50% and 20% of 1461 cells, respectively. Furthermore, specific uptake and degradation by megalin/gp330-expressing cells was inhibitable by RAP to a residual 40% to 50%, whereas on megalin/gp330-deficient control cells, uptake and degradation occurred via pathways that were clearly not RAP-sensitive (Figure 4B and 4C).

Owing to the biological variability that is inherent to experimental systems, the absolute values varied considerably for all radioactive assays. However, the relative differences as expressed in the figures were observed consistently, irrespective of the absolute values.

Cellular Uptake of Dil-Fluorescence–Labeled Lp(a)

Fluorescence studies confirmed the results obtained with ¹²⁵I-radiolabeled Lp(a), in that uptake of Dil-labeled Lp(a) appeared to be much more efficient on 1461 cells than on 6A3 controls. Immunofluorescence incubations of the 2 cell lines with a polyclonal anti-megalin/gp330 antibody resulted in a distinct punctate signal on 1461, whereas it yielded only a background stain on 6A3 cells (green in Figure 5A and 5D). For fluorescence tracing of endocytosed ligands, we used 100 pmol/mL Dil-Lp(a). Uptake was allowed to continue for 20 minutes at 37°C, resulting in a clear-cut difference of intensity in the red fluorescence signal originating from internalized Dil-Lp(a) between the 2 cell lines. A perinuclear endosomal staining pattern was easily detectable on 1461 cells, whereas under the same conditions, 6A3 cells took up hardly any Dil-Lp(a) (Figure 5B and 5E). Superimposition of images 5A and 5B as well as of 5D and 5E demonstrates that significant uptake of Lp(a) occurred only on megalin/gp330-expressing cells (Figure 5C and 5F).

Direct Binding of Lp(a) to Immobilized Megalin/gp330

To obtain experimental evidence for a direct molecular interaction between megalin/gp330 and Lp(a) as suggested by
the results of the cell assays, we analyzed the binding of Lp(a) to megalin/gp330 and the LDLR and compared it to the binding of equimolar amounts of LDL (BIAcore system). Purified receptors were immobilized on flow-cell sensor chips at a concentration of 17 (for megalin/gp330) and 19 (for LDLR) fmol/mm², and flow cells were injected with equimolar amounts of Lp(a) and LDL over a concentration range of 10 to 100 pmol/mL, corresponding to the concentrations used in the various cell assays. As shown in Figure 6A, Lp(a) as well as LDL strongly bound to megalin/gp330 in a calcium-dependent manner. The binding of Lp(a) (Kₐ 1.5 nmol/L) occurred with slightly higher affinity than did LDL binding (Kₐ 3.4 nmol/L). Binding of both Lp(a) and LDL was abolished by withdrawal of calcium (ie, in the presence of EDTA). For the LDLR, the difference between LDL and Lp(a) binding was much more pronounced: LDL bound strongly to the LDLR (Kₐ 1.9 nmol/L) in the presence but not in the absence of calcium. In contrast to LDL, Lp(a) binding to the LDLR was hardly measurable and was independent of the presence of calcium.

**Cellular Uptake of Lp(a) and LDL by Wild-Type and LDLR-Negative Fibroblasts**

The BIAcore data show that Lp(a) can bind, if only very weakly, to the LDLR. Because both cell lines 1461 and 6A3 express the LDLR to some extent, we therefore aimed to rule out potential interference from the LDLR to the Lp(a) uptake and degradation assays with 1461 and 6A3 cells. We therefore performed uptake experiments with cells more suitable for analyzing the functional importance of the LDLR. Wild-type human fibroblasts were compared with LDLR-negative fibroblasts derived from a patient with familial hypercholesterolemia (FH) with regard to their ability to take up ¹²⁵I-LDL and ¹²⁵I-Lp(a). As expected, a drastic difference in the uptake of LDL between wild-type and familial hypercholesterolemia fibroblasts was observed (Figure 7); however, uptake of Lp(a) did not differ much and was considerably lower, reaching 30% of LDL uptake at maximum. These data reflect and confirm the low affinity of Lp(a) to the LDLR (cf Figure 6B).

**Cross-Competition of Lp(a) and LDL in Megalin/gp330-Mediated Cellular Uptake**

The cellular uptake of Lp(a) via megalin/gp330 could be mediated by either apo(a) or apoB100 binding to the receptor. Because apoB100 has been previously described as a ligand for megalin/gp330 before, we investigated whether the cellular uptake of ¹²⁵I-Lp(a) could be inhibited by an excess of unlabeled LDL and vice versa. First, we compared the uptake of ¹²⁵I-LDL by the cell lines 1461 and 6A3. These assays were performed under the same experimental conditions as the Lp(a) uptake assays (cf Figure 4) and yielded surprisingly similar results (Figure 8A). LDL uptake by megalin/gp330-expressing 1461 cells was RAP-sensitive and twice as high, on average, as uptake by the control cells, which in turn was not inhibitable by the addition of RAP. These results suggest that a similar or an identical cellular mechanism is responsible for the internalization of LDL and Lp(a), a finding that is in agreement with the comparable binding of both ligands to purified megalin/gp330 (Figure 6A). Finally, the addition of a 50-fold molar excess of unlabeled Lp(a) and LDL to the incubation medium with either ¹²⁵I-Lp(a) or ¹²⁵I-LDL resulted in a similar pattern of inhibition for both ligands on megalin/gp330-expressing 1461 cells (Figure 8B). In the case of ¹²⁵I-LDL uptake, owing to the presence of the LDLR, the inhibition by LDL was more efficient than that by Lp(a).

**Discussion**

In this study a cell system derived from LRP- and megalin/gp330-knockout mice embryos was used for the functional analysis of megalin/gp330 with regard to its ability to bind and mediate the cellular uptake and degradation of Lp(a) in vitro. ApoB100 has been previously described as a ligand for megalin/gp330; however, receptor binding of apoB100 does not necessarily imply that the respective receptor will bind Lp(a), as shown for the LDLR, which has been under discussion as a potential Lp(a) receptor in numerous studies. Megalin/gp330 is known to be the only member of the LDLR gene family to bind the apo(a) homologue plasminogen, thus suggesting that Lp(a) might bind to megalin/gp330 by either apoB100, apo(a), or both apolipoproteins. Taking different methodological approaches, we compared the interaction of Lp(a) and LDL with megalin/gp330 and the LDLR and demonstrated that megalin/gp330 constitutes a specific receptor for Lp(a) in vitro. We also confirmed previous data obtained by several groups, including our own, that came to the conclusion that the LDLR is able to bind Lp(a) but that the interaction is weak and of a smaller order of magnitude than that of the LDL-LDLR interaction. A mouse embryonic yolk sac cell line with native expression of megalin/gp330 but genetically deficient for LRP was compared with a control cell line carrying a double knockout for both megalin/gp330 and LRP. We made use of these 2 cell lines to examine whether megalin/gp330 was capable of binding and mediating the cellular uptake and degradation of Lp(a). Lp(a) was specifically taken up and degraded by 1461 cells in a saturable manner, with average values being twice as high as for control cells. The pathway on 1461 cells was sensitive to the addition of RAP, a potent inhibitor of ligand binding to megalin/gp330, suggesting that the difference between the cell lines was mediated by megalin/gp330. It is of note that there was specific uptake and degradation of Lp(a) by the control cell line, occurring via a pathway that was not RAP-sensitive and for which the mechanism remains unclear at present. However, this did not have any bearing on the presumably megalin/gp330-mediated difference between the cell lines. To confirm the results obtained with ¹²⁵I-Lp(a) and to rule out potential artifacts inherent to the radiolabel, we followed the fate of DiI-labeled Lp(a) by means of fluorescence microscopy and again observed a difference in uptake between the cell lines. The indirect evidence for Lp(a) interaction with gp330/megalin obtained from the cell studies was further elaborated by binding studies investigating the direct molecular interaction of Lp(a) with immobilized, purified megalin/gp330. Experiments employing the BIAcore system enabled us to quantify the binding of equimolar amounts of Lp(a) and LDL to immobilized megalin/gp330 and LDLR, revealing that binding of Lp(a) to megalin/gp330 occurred with similar affinity as LDL binding. The binding was calcium dependent in both cases. In contrast, binding of Lp(a) to the LDLR was hardly measurable, providing further evidence that Lp(a) constitutes a poor ligand for the LDLR. However, because the yolk sac cell line 1461
was shown to express slightly higher amounts of LDLR than did control cells (Figure 1B), it was essential to make sure that the weak Lp(a) binding to the LDLR was of negligible influence within the assay system used in this study. Therefore, LDLR-negative FH fibroblasts were used. Uptake of Lp(a) was only \( \approx 30\% \) less than by wild-type fibroblasts, demonstrating that even a functional null mutation of the LDLR gene does not prove differences as high as those observed between the yolk sac cell lines. Therefore, the difference in uptake and degradation of Lp(a) between the yolk sac cell lines can only be explained by megalin/gp330.

In this study, isoform 21 was used as a standard for all experimental approaches. In cellular assays, other isoforms behaved similarly, suggesting that the binding to megalin/gp330 is not an isoform-specific phenomenon. However, whether this holds true for the entire spectrum of apo(a) isoforms remains unknown at this point and will have to be elucidated by a broader, more systematic approach. Uptake experiments with \(^{125}\)I-LDL revealed the same RAP-sensitive difference between megalin/gp330-expressing and control cell lines as observed for \(^{125}\)I-Lp(a). Because apoB100 has been described as a ligand for megalin/gp330 before,\(^4,44\) this finding was not surprising but raised the question as to the mechanism of Lp(a) binding to megalin/gp330. From the displacement studies with unlabeled Lp(a) and LDL, showing that the megalin/gp330-mediated uptake of Lp(a) was competitively inhibitable by both Lp(a) and LDL, we conclude that the megalin/gp330-Lp(a) interaction involves apoB100.

Taking into account that the apo(a) homologue plasminogen binds to megalin/gp330,\(^3\) it is still tempting to speculate that apo(a) might be partially responsible for the mediation of Lp(a) binding, perhaps in a concerted action with apoB100. However, the addition of plasminogen alone did not have any effect on Lp(a) uptake by either cell line (data not shown).

Based on these data, further studies will be needed to completely elucidate the nature of the binding mechanism of Lp(a) to megalin/gp330. At its epithelial expression sites in vivo, megalin/gp330 faces a specialized milieu, such as the cerebrospinal fluid, seminal fluid, or primary urinary filtrate.\(^46,49,50\) As of yet, there are no reports of the existence of intact Lp(a) in these fluids. Therefore, the question whether megalin/gp330-mediated uptake of Lp(a) can play a physiologically important role remains open at present. To address these questions, further investigations will clearly be needed.

In conclusion, we have identified megalin/gp330 as a lipid-binding receptor responsible for the internalization of lipoprotein(a) in cultured human cells. Our findings also suggest a potential role of megalin/gp330 in the development of atherosclerosis. Further studies are needed to clarify the mechanism by which megalin/gp330 binds lipoprotein(a)

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