Mesd binds to mature LDL-receptor-related protein-6 and antagonizes ligand binding

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Summary

Wnt co-receptors LRP5 and LRP6 are two members of the low-density lipoprotein receptor family. Receptor-associated protein is not only a specialized chaperone but also a universal antagonist for members of the low-density lipoprotein receptor family. Here we test whether Mesd, a newly identified chaperone for members of the low-density lipoprotein receptor family, also binds to mature receptors at the cell surface and antagonizes ligand binding. We found that Mesd binds to cell surface LRP5 and LRP6, but not to other members of the low-density lipoprotein receptor family. Scatchard analysis revealed that Mesd binds cell surface LRP6 with high affinity ($K_d \sim 3.3$ nM). Interestingly, the C-terminal region of Mesd, which is absent in sequences from invertebrates, is necessary and sufficient for binding to mature LRP6, and is required for LRP6 folding. We also found that LRP6 is not a constitutively active endocytosis receptor and binding of the receptor-associated protein to LRP6 partially competes for Mesd binding. Finally, we demonstrated that Mesd antagonizes ligand binding to LRP6 at the cell surface. Together our results show that in addition to serving as a folding chaperone, Mesd can function as a receptor antagonist by inhibiting ligand binding to mature LRP6.

Key words: LRP6, LDLR family, Wnt signaling, Mesd, RAP, DKK1

Introduction

The canonical Wnt signaling pathway is involved in various differentiation events during embryonic development and can lead to tumor formation when aberrantly activated (Orford et al., 1997; Wodarz and Nusse, 1998; Giles et al., 2003; Lustig and Behrens, 2003; He et al., 2004). The low-density lipoprotein-receptor (LDLR)-related-protein-5 (LRP5) and LRP6 are two members of the expanding LDLR family (Herz et al., 1997; Wodarz and Nusse, 1998; Giles et al., 2003; Lustig and Behrens, 2003; He et al., 2004). Recent studies have demonstrated that these two receptors are indispensable elements of the canonical Wnt pathway by interacting with several components of the Wnt signaling pathway. The extracellular domain of LRP5/6 can bind at least four types of secreted proteins including Wnts, Dickkopfs (DKKs), Wise, and the connective-tissue growth factor (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; Wu et al., 2000; Bafico et al., 2001; Bo et al., 2001; Semenov et al., 2001; Brott and Sokol, 2002; Schneider and Nimpf, 2003; He et al., 2004). The cytoplasmic domains of LRP5/6, upon receptor activation by Wnt proteins, recruit the cytosolic scaffold protein axin to the membrane, therefore preventing it from participation in the degradation of β-catenin (Mao et al., 2001; Liu et al., 2003; Tolwinski et al., 2003; Tamai et al., 2004). Recently, it was reported that a PPPSP motif, which is reiterated five times in the LRPs/6 cytoplasmic tail, is necessary and sufficient to trigger Wnt/β-catenin signaling (Brennan et al., 2004; Tamai et al., 2004). LRP6-deficient mice are perinatal lethal and exhibit mid/hindbrain defects, posterior truncation and abnormal limb patterning. These phenotypes are similar to those observed in mice carrying mutations in Wnt genes, specifically Wnt1, Wnt3a and Wnt7a (Pinson et al., 2000). LRP5-deficient mice have normal embryogenesis, grow to adulthood and are fertile, but show defects in bone accrual, eye development, and lipoprotein and glucose metabolism (Kato et al., 2002; Fujino et al., 2003; Magoori et al., 2003).

The 39 kDa receptor-associated protein (RAP) is a specialized molecular chaperone for the members of the LDLR family, and functions intracellularly as a molecular chaperone to facilitate receptor folding and trafficking within the early secretory pathway (Bu, 2001). RAP is also a unique receptor antagonist for members of the LDLR family. RAP, which binds with high affinity to LRP, megalin, very-low-density lipoprotein receptor (VLDLR), and apolipoprotein E receptor 2 (apoER2), and with a lower affinity to the LDLR, is able to inhibit the binding of most currently known ligands of LDLR family members (Bu, 2001).

More recently, two groups have identified a novel specialized chaperone for members of the LDLR family, termed Mesd (mesoderm development in mouse (Hsieh et al., 2003) and Boca in Drosophila (Culi and Mann, 2003). This new chaperone (195 amino acids in mouse) was discovered due to its requirement for the folding of LRP5/LRP6, co-receptors...
for the Wnt/Wg signaling pathway. In the mouse, the consequences of Mesd deficiency resemble that seen in wnt3-deficient mutants. However, the presence of more extensive defects also in extra embryonic tissues as well as abnormal cell proliferation in the epiblast of Mesd-deficient embryos suggests that signals involving either other Wnt proteins or other LDLR family members are also affected.

Similarly to other ER chaperones, Mesd also carries an ER retention signal (KDEL in Drosophila, REDL in mammals) at its C-terminus and localizes to the ER by immunohistochemistry (Iadonato et al., 1993; Culi and Mann, 2003). All members of the LDLR family have at least one six-bladed β-propeller domain, which is immediately followed by an epidermal growth factor (EGF) repeat. Mesd is specifically required for the maturation of these β-propeller/EGF modules through the secretory pathway (Culi et al., 2004). In the absence of Mesd, LRP5/LRP6 form aggregates in the ER and fail to reach the cell surface.

In the present studies, we tested whether Mesd, like RAP, also binds to cell surface receptors, and antagonizes ligand binding of the LDLR family members. Our data show that Mesd is capable of binding mature LRP6 at the cell surface and antagonizing ligand binding.

Materials and Methods

Materials

Human recombinant DKK1 protein and mouse recombinant Wnt3a protein were from R&D Systems. Human recombinant RAP protein was expressed in a glutathione S-transferase (GST) expression vector and isolated as described previously (Bu et al., 1993). Monoclonal anti-Myc antibody 9E10 was from Roche. Monoclonal antibody 8G11 against human LRP was from Research Diagnostics. Monoclonal anti-HA antibody has been described before (Li et al., 2000). Polyclonal rabbit anti-LDLR was produced by immunizing rabbits with recombinant human LDLR1-294 fragment. Peroxidase-labeled antiaqu mouse antibody and ECL system were from Amersham Life Science. Plasmid pcDNA3.1-Myc-hLRP5 containing the full-length human LRP5 cDNA and plasmid pCS-Myc-hLRP6 containing the full-length human LRP6 cDNA were from Cindy Bartels (Case Western Reserve University, Cleveland, OH) and Christof Niehrs (Deutsches Kresforschungszentren, Heidelberg, Germany), respectively. Carrier-free Na125I was purchased from NEN Life Science Products. IODO-GEN was from Pierce. Proteins were iodinated by using the IODO-GEN method as described previously (Li et al., 2000).

Cell lines and cell culture

LRP6-transduced HT1080 cells and the control cells have been described before (Li et al., 2004), and were cultured in DMEM medium containing 10% fetal bovine serum and 350 μg/ml G418. The LRP-null CHO cells stably transfected with human LDLR-related protein (LRP) minirepressor mLRP4, mLRP4 tail mutant mLRP4tailless (mLRP4 without the cytoplasmic tail), human LDLR-related protein 1B (LRP1B) minirepressor mLRP1B4, human VLDLR, or human apoER2 have been described before (Li et al., 2000; Li et al., 2001; Liu et al., 2001), and were cultured in Ham’s F-12 medium containing 10% fetal bovine serum and 350 μg/ml G418. A set of genetically derived murine embryonic fibroblasts (MEF) from mouse embryos deficient for LRP and/or LDLR were obtained from Joachim Herz, University of Texas Southwestern Medical Center at Dallas (Willnow and Herz, 1994; Narita et al., 2002). These are MEF-1 (WT), MEF-2 (LRP-deficient), MEF-3 (LDLR-deficient), and MEF-4 (LRP and LDLR-double-deficient), and are cultured in DMEM containing 10% fetal bovine serum. Culture conditions of U87, MCF-7, and human aortic smooth muscle cells have been described before (Li et al., 2003). HEK293 cells were from ATCC, and cultured in DMEM containing 10% fetal bovine serum.

Preparation of recombinant Mesd protein

Full-length mouse Mesd cDNA was kindly provided by Bernardette Holdener (State University of New York at Stony Brook, Stony Brook, NY). The wild-type and mutant forms of mouse Mesd were generated by polymerase chain reactions, and subcloned into the expression vector pET-30a(+) (Novagen) at the EcoRI and HindIII restriction sites. The integrity of the subcloned DNA sequence was confirmed by DNA sequencing. Recombinant proteins were overexpressed from pET-30a(+)/Mesd in E. coli. BL21(DE3) producing a recombinant fusion protein with a polyhistidine metal-binding tail at the N-terminus, and purified with His-Bind Kits from Novagen according to the manufacturer’s protocol. All the recombinant Mesd proteins lack the Mesd signal peptide.

Western blotting

To examine the expression of the LDLR family members, cells cultured in six-well plates were lysed with 0.5 ml lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM PMSF) at 4°C for 30 minutes. Equal quantities of protein were subjected to SDS-PAGE under non-reducing conditions. Following transfer to Immobilon-P membrane, successive incubations with primary antibody and horseradish peroxidase-conjugated secondary antibody were carried out for 60 minutes at room temperature. The immunoreactive proteins were then detected using the ECL system.

To examine the cytosolic β-catenin level, cells in six-well plates were treated with Mesd at various concentrations for 90 minutes at 37°C. After washing in ice-cold PBS, cells were collected and homogenized in a glass Dounce homogenizer in buffer consisting of 100 mM Tris-HCl pH 7.4, 140 mM NaCl, 2 mM DTT, 2 mM PMSF, and 1× Complete™ protease inhibitors (500 μl/well). The homogenate was centrifuged for 10 minutes at 500 g, and the supernatant was further centrifuged at 100,000 g at 4°C for 90 minutes. The resulting supernatant was designated the cytosolic fraction. The β-catenin levels were then examined by western blotting using β-catenin-specific antibody from Cell Signaling Technology. The immunoreactive proteins were detected using the ECL system. Films showing immunoreactive bands were scanned with a Kodak Digital Science DC120 Zoom Digital Camera and band intensities were analyzed with Kodak Digital Science1D Image Analysis Software.

Luciferase reporter assay

HEK293 cells were plated into six-well plates. For each well, 0.1 μg of the TOP-FLASH TCF luciferase construct (Upstate Biotechnology) was cotransfected with 0.8 μg Mesd-expressing vector, 0.8 μg Mesd mutant-expressing vector, or empty vector. A β-galactosidase-expressing vector (Promega, Madison, WI) was included as an internal control for transfection efficiency. After 48 hours, cells were lysed and both luciferase and β-galactosidase activities were determined with enzyme assay kits (Promega). The luciferase activity was determined with a luminometer using the Dual Luciferase Assay system (Promega). Luciferase activity was normalized to the activity of the β-galactosidase.

Ligand binding and degradation

Cells (2×105) were seeded into 12-well dishes 1 day prior to assay. Ligand-binding buffer (minimal Eagle’s medium containing 0.6% BSA with a different concentration of radioligand, 0.6 ml/well) was added to cell monolayers, in the absence or the presence of 500 nM
unlabeled RAP or 500 nM unlabeled Mesd, followed with incubation for 0–4 hours at 4°C. Thereafter, overlying buffer containing unbound ligand was removed, and cell monolayers were washed and lysed in low-SDS lysis buffer (62.5 mM Tris–HCl pH 6.8, 0.2% SDS, 10% v/glycerol) and counted. The protein concentration of each cell lysate was measured in parallel dishes that did not contain the ligands.

Ligand degradation was performed using the methods as described (Li et al., 2000). Briefly, 2×10^5 cells were seeded into 12-well dishes 1 day prior to assay. Pre-warmed assay buffer (minimal Eagle’s medium containing 0.6% BSA with radioligand, 0.6 ml/well) was added to cell monolayers in the absence or the presence of unlabeled 500 nM RAP or 500 nM Mesd, followed by incubation for 4 hours at 37°C. Thereafter, the medium overlying the cell monolayers was removed and proteins were precipitated by addition of BSA to 10 mg/ml and trichloroacetic acid to 20%. Degradation of radioligand was defined as the appearance of radioactive fragments in the overlying medium that were soluble in 20% trichloroacetic acid.

**Kinetic analysis of endocytosis**

LRP6-transduced HT1080 cells were plated in 12-well plates at a density of 2×10^5 cells/well and used after overnight culture. Cells were rinsed twice in ice-cold assay buffer (minimal Eagle’s medium containing 0.6% BSA), and 125I-anti-HA IgG was added at 1 nM final concentration in cold assay buffer (0.5 ml/well). The binding of 125I-anti-HA IgG was carried out at 4°C for 90 minutes with gentle rocking. Unbound 125I-anti-HA IgG was removed by washing cell monolayers three times with cold assay buffer. Ice-cold stop/strip solution (0.2 M acetic acid, pH 2.6, 0.1 M NaCl) was added to one set of plates without warming up and kept on ice. The remaining plates were then placed in a 37°C water bath and 0.5 ml assay buffer prewarmed to 37°C was quickly added to cell monolayers to initiate internalization. After each time point, the plates were quickly placed on ice and the assay buffer was replaced with cold stop/strip solution. 125I-anti-HA IgG that remained on the cell surface was stripped by incubation of cell monolayers with cold stop/strip solution for a total of 20 minutes (0.75 ml for 10 minutes, twice) and counted. Cell monolayers were then solubilized with low-SDS lysis buffer and counted. The sum of 125I-anti-HA IgG that was internalized plus that remaining on the cell surface after each assay was used as the maximum potential internalization. The fraction of internalized 125I-anti-HA IgG after each time point was calculated and plotted.

**Cell surface DKK1 binding and immunodetection**

Human DKK1 cDNA (clone MGC:868, IMAGE:3508222) was obtained from Invitrogen and subcloned into pcDNA3 (EcoRI/XbaI). To facilitate immunodetection, a c-Myc epitope was included at the C-terminus. The integrity of the subcloned DNA sequence was confirmed by DNA sequencing. Human DKK1-conditioned media were produced by transient transfection of HEK293 cells with pcDNADKK1-Myc in serum-free medium, and allowed to bind to LRP6-transduced HT1080 cells and control cells at room temperature for 60 minutes in the absence or presence of 1 μM Mesd. Cells were then fixed in 4% paraformaldehyde, labeled with anti-Myc monoclonal antibody and detected with Alexa-488 goat anti-mouse IgG. The immunofluorescence was detected by a laser-scanning confocal microscope (Olympus Fluoview 500).

**Results**

**Mesd binds to mature LRP6 at the cell surface**

The specialized molecular chaperone RAP binds with high affinity to most members of the LDLR family at the cell surface. To examine whether Mesd also possesses this feature, we performed cell surface ligand binding experiments with cells stably transduced with LRP6 cDNA. Human HT1080 cells, which express undetectable levels of LRP6, were transduced with a viral vector alone (pLNCX2) or with vector containing LRP6 cDNA (Li et al., 2004) and used for 125I-Mesd binding (Fig. 1A). 125I-Mesd (5 nM) reached maximal binding after 2 hours incubation at 4°C with LRP6-expressing HT1080 cells (Fig. 1A). Inclusion of excess unlabeled Mesd (500 nM) completely eliminated this binding. No significant 125I-Mesd binding was seen with the control cells (pLNCX2). Saturation of Mesd specific binding was seen at concentrations of >6.4 nM (Fig. 1B). Scatchard analysis of the binding data revealed that Mesd binds LRP6 with a K_d of ~3.3 nM (Fig. 1C). This affinity of Mesd to LRP6 is comparable to that of RAP to LRP (Iadonato et al., 1993).

To determine whether Mesd binds to other members of the LDLR family, we performed 125I-Mesd binding analysis with four groups of cells expressing different members of the LDLR family (Fig. 2). In the first experiment, we used HEK293 cells transiently transfected with cDNAs for the LDLR, LRP5,
LRP6 or empty pcDNA3 vector. In the second experiment, we used LRP-null Chinese hamster ovary (CHO) cells stably transfected with LRP minireceptor mLRP4, mLRP1B minireceptor mLRP1B4, VLDLR, apoER2 or empty pcDNA3 vector only. (C) Binding of 125I-Mesd (5 nM) to wild-type murine embryonic fibroblasts (MEF-1) or MEF cell lines genetically deficient in LRP (MEF-2), LDLR (MEF-3) or both (MEF-4). Lower panel, western blot analysis of LRP and the LDLR expression in MEF cell lines. (D) Binding of 125I-Mesd (5 nM) to human breast cancer cell line MCF-7, human glioblastoma cell line U87 and human aortic smooth muscle cells (SMC). Lower panel, western blot analysis of LRP expression in these cell lines. Assays were carried out for 4 hours at 4°C in the absence (total) or presence of 500 nM Mesd. Values are the means of triple determinations with the s.d. indicated by error bars. *P<0.01 indicates a significant difference compared with control cells transfected with empty pcDNA3 vector.

The C-terminal region of Mesd is necessary and sufficient for LRP6 binding

Having established that Mesd binds to mature LRP6 at the cell surface with high affinity, we then investigated the Mesd sequences that are required for this interaction. Upon sequence comparison of mouse Mesd and its homologs from different species, we found that the first 12 amino acids of mouse Mesd are absent in Caenorhabditis elegans and Caenorhabditis briggsae, and that mouse Mesd, as well as human Mesd, has an extra ~30 amino acid fragment prior to the conserved ER retention signal in its C-terminus (Culi and Mann, 2003; Hsieh et al., 2003). We thus generated two truncated Mesd mutants lacking either the N-terminal region, MESD(12-195), or both the N-terminal and C-terminal regions, Mesd(12-155) (Fig. 3A). The ability of these mutants to bind to cell surface LRP6 was then assessed. Interestingly, we found that although truncation of the N-terminal 11 amino acids of mouse Mesd had no effect on LRP6 binding, further truncation of the last 40 amino acids completely abolished LRP6 binding (Fig. 3B). Therefore, specific binding of Mesd to CHO and MEF cells may reflect endogenous LRP5/LRP6 in these cells.

Fig. 2. Mesd binds to mature LRP5/6 but not significantly to other members of the LDLR family. (A) Binding of 125I-Mesd (5 nM) to HEK293 cells transiently transfected with human HA-tagged LDLR, Myc-tagged LRP5, Myc-tagged LRP6 or control vector. Lower panel, western blot analysis for the expression of the LDLR, LRP5 and LRP6. Equal amounts of cell lysate were applied for each lane. (B) Binding of 125I-Mesd (5 nM) to LRP-null CHO cells stably transfected with LRP minireceptor mLRP4, LRP1B minireceptor mLRP1B4, VLDLR, apoER2 or empty pcDNA3 vector only. (C) Binding of 125I-Mesd (5 nM) to wild-type murine embryonic fibroblasts (MEF-1) or MEF cell lines genetically deficient in LRP (MEF-2), LDLR (MEF-3) or both (MEF-4). Lower panel, western blot analysis of LRP and the LDLR expression in MEF cell lines. (D) Binding of 125I-Mesd (5 nM) to human breast cancer cell line MCF-7, human glioblastoma cell line U87 and human aortic smooth muscle cells (SMC). Lower panel, western blot analysis of LRP expression in these cell lines. Assays were carried out for 4 hours at 4°C in the absence (total) or presence of 500 nM Mesd. Values are the means of triple determinations with the s.d. indicated by error bars. *P<0.01 indicates a significant difference compared with control cells transfected with empty pcDNA3 vector.
Mesd binds to mature LRP6

Mesd is a specific chaperone for LRP5 and LRP6. To examine the role of this C-terminal region of Mesd on receptor folding, we generated a Mesd mutant (MesdΔC), which lacks the C-terminal region (amino acids 156-191) but retains the ER retention signal (REDL) (Fig. 3A). We next evaluated a potential role for MesdΔC on LRP6 folding. HEK293 cells were transiently transfected with cDNA for the LRP6 with cotransfection of control vector, or cDNAs for Mesd or MesdΔC. The steady-state levels of LRP6 were analyzed by western blotting with the anti-MYC antibody (Fig. 5A). As seen in the figure, two forms of the receptor, i.e. the ER form and mature form (containing complex sugar modifications), were seen for LRP6. In the presence of Mesd coexpression, but not of MesdΔC coexpression, the amount of the mature form of LRP6 was significantly increased (Fig. 5A).

Activation of canonical Wnt signaling leads to the stabilization of β-catenin and regulation of gene transcription through transcription regulators including lymphoid-enhancing factor (LEF)-1 and T-cell factors (TCF). The TOP-FLASH luciferase reporter contains TCF-binding sites and can be directly activated by the β-catenin/TCF complex (Korinek et al., 1997). LRP6 is cell surface receptor, and only the mature receptor can reach the cell surface and modulate Wnt signaling (Cong et al., 2004). We next examined the effect of MesdΔC on Wnt signaling using the TOP-FLASH luciferase reporter assay in HEK293 cells. As expected, Mesd coexpression, but not MesdΔC coexpression, significantly enhanced TCF/LEF transcriptional activity (Fig. 5B). Together, these results suggest that the C-terminal region of Mesd is required for LRP6 folding and its signaling function at the cell surface.

LRP6 is not a constitutively active endocytosis receptor and mediates a limited level of Mesd degradation

Cell surface receptors that traffic between the plasma membrane and endocytic compartments contain signals within their cytoplasmic tails that allow for efficient recruitment into endocytic vesicles. In many cases (e.g. LRP and the LDLR), these signals are constitutively active and mediate continuous receptor endocytosis independently of ligand binding. To examine whether LRP6 is a constitutively active endocytosis receptor, we performed kinetic analysis of receptor endocytosis with HT1080 cells transduced with HA-tagged LRP6. To eliminate potential effects of LRP6 ligands on its...
内部化，我们利用125I-anti-HA IgG进行LRP6内吞作用的实验。125I-anti-HA IgG与HA标签的LRP6结合是特定的，即与不含LRP6的HT1080对照细胞相比，HT1080-LRP6细胞的结合量显著增加（图6A）。我们使用HA标签的LRP4作为阳性对照，和mLRP4tailess（缺乏胞浆尾）作为阴性对照，进行125I-anti-HA IgG内吞作用的实验（Li et al., 2000）。有趣的是，我们发现LRP6的内吞速率非常慢，并且与mLRP4tailess相近（图6B），这表明LRP6本身不能启动内吞作用。

在证明Mesd与细胞表面LRP6有高亲和力，并且LRP6在无配体刺激的情况下不能启动内吞作用后，我们研究了LRP6介导的Mesd内化和降解。HT1080细胞转导的LRP6在4小时的37°C孵育后，125I-Mesd的降解水平为320 fmoles/mg cell protein，而在4°C孵育4小时后，125I-Mesd的结合量高达1320 fmoles/mg cell protein（图7）。这些结果表明Mesd与细胞表面LRP6的结合不会触发显著的内吞作用，因此很少的Mesd内化和降解可以检测。

Mesd与细胞表面LRP6的结合不会显著改变胞质中β-catenin的水平。

β-catenin是Wnt/β-catenin信号通路中的一个关键分子，与DNA结合蛋白相互作用，并参与Wnt信号转导（Hinck et al., 1994; Gottardi et al., 2001; Klingelhofer et al., 2003）。为了确定Mesd与细胞表面LRP6的结合是否直接调节Wnt信号，我们研究了Mesd与细胞表面LRP6结合对胞质中β-catenin水平的影响。因此，我们用0.5到5 nM Mesd处理HT1080-LRP6细胞2小时，然后通过Western Blotting检测胞质中β-catenin水平。我们发现Mesd处理后，β-catenin水平没有显著变化（数据未显示），这表明Mesd与细胞表面LRP6的结合不直接调节Wnt信号。

RAP与LRP6结合，部分竞争Mesd的结合。

RAP以高亲和力结合到LRP，megalin，VLDLR和apoER2，对LDLR的亲和力较低（Bu, 2001）。为了确定是否RAP也结合到LRP6，我们进行RAP-竞争实验。控制细胞，即空载体转导的细胞，具有中等程度的细胞表面125I-RAP结合，可能是由细胞表面的硫酸酸性蛋白聚糖和内源性LDLR家族受体介导的。添加过量的无标记RAP（500 nM），但不添加Mesd（500 nM），完全消除了这种结合（图8A）。与对照细胞相比，LRP6转导的HT1080细胞的RAP结合量增加了约20%，这个增加被过量Mesd（500 nM）完全阻断（图8A）。这些结果表明RAP与细胞表面LRP6的结合具有相对低的亲和力。

为了确定RAP和Mesd是否结合到LRP6上相同的、重叠的或不同的位点，我们进行了RAP和Mesd的结合竞争实验。RAP结合到细胞表面LRP6，具有相对低的亲和力。
Mesd binds to mature LRP6

500 nM unlabeled Mesd (Fig. 8B). RAP inhibited $^{125}$I-Mesd binding in a dose-dependent manner with ~60% inhibition achieved with 500 nM RAP, whereas the same concentration of unlabeled Mesd inhibited >90% of $^{125}$I-Mesd binding (Fig. 8B). When $^{125}$I-Mesd (5 nM) uptake and degradation were performed, 500 nM unlabeled Mesd completely, whereas 500 nM unlabeled RAP only partially, inhibited $^{125}$I-Mesd degradation (Fig. 8C). Together, these results suggest that Mesd and RAP probably bind to different, but perhaps adjacent sites on LRP6. The lower affinity of RAP to cell surface LRP6 may also contribute to its lower efficiency in inhibition of Mesd binding.

Mesd antagonizes ligand binding to LRP6 at the cell surface

RAP is a unique receptor antagonist for members of the LDLR family, and is able to inhibit the binding of most known ligands of the LDLR family members. To determine whether Mesd is also able to block LRP6 ligand binding, we examined cell surface DKK1 binding by immunostaining. DKK1 is an LRP6-specific ligand and antagonist. As expected, Myc-tagged DKK1 binds to LRP6 cells (Fig. 9B) but not to the control cells (Fig. 9A). Importantly, the presence of Mesd completely blocked the binding of Myc-DKK1 to LRP6 at the cell surface (Fig. 9C).

To confirm the above results, we examined the binding and degradation of $^{125}$I-DKK1. LRP6-expressing HT1080 cells exhibited significantly higher levels of $^{125}$I-DKK1 binding and degradation than the control cells. The increased DKK1 binding and degradation were abolished by excess unlabeled Mesd, but not by excess unlabeled RAP (Fig. 9D,E). Together, these results indicate that Mesd can specifically block DKK1 binding to LRP6 at the cell surface.

**Discussion**

Mesd binds to LRP5 and LRP6 at the cell surface

Recent studies have identified Mesd as a novel ER chaperone that is specifically required for the correct folding and trafficking of several members of the LDLR family including LRP5, LRP6, LDLR and LRP (Culi and Mann, 2003; Hsieh et al., 2003; Culi et al., 2004). However, flies or mice with a nonfunctional Boca or Mesd gene die during embryogenesis and display phenotypes that are consistent with an inability to transduce a Wg/Wnt signal, suggesting that Mesd is probably more important for LRP5/6 than for other members of the receptor folding, trafficking, and maturation. Thus, it is not surprising as demonstrated here that Mesd binds to cell surface...
LRP5 and LRP6, but not to other members of the LDLR family.

Although mouse Mesd protein shares ~45% identity and ~65-85% similarity with open reading frames predicted in Mesd from different species, mouse Mesd as well as human Mesd has an extra ~30 amino acid fragment prior to the conserved C-terminal ER-retention signal (Culi and Mann, 2003; Hsieh et al., 2003). In the present study, we found that this C-terminal region, uniquely present in vertebrate Mesd, is necessary and sufficient for binding to mature LRP6 at the cell surface. We also found that this region is required for LRP6 folding. It is tempting to speculate that the ability of Mesd to bind mature LRP6 and regulate ligand binding/Wnt signaling might be a unique feature of vertebrate Mesd acquired during evolution. Because fly Mesd (termed Boca) (Culi and Mann, 2003), despite lacking this C-terminal region, is also essential for the folding of arrow (the ortholog of LRP5 and LRP6 in Drosophila), the mechanisms of Mesd chaperone function might have also evolved during evolution. The biological significance of our findings requires further investigation.

**RAP binds to LRP6**

To date, the most dramatic effect observed when RAP is bound to LRP is the inhibition of binding and/or uptake of all known LRP ligands. This ability of RAP distinguishes it from other LRP ligands, which seldom inhibit the binding and/or uptake of each other. In addition to LRP, RAP also binds to other members of the LDLR gene family and inhibits ligand interactions. Studies have shown that RAP exhibits high-affinity binding ($K_D \sim 1-10$ nM) for LRP, megalin, the VLDLR and apoER2, but low affinity ($K_D \sim 250$ nM) for the LDLR (Bu, 2001). In the present study, we demonstrated that RAP binds to LRP6 at a relatively low affinity and that RAP binding to LRP6 can be blocked by Mesd. We also demonstrated that RAP can partially compete for Mesd binding to LRP6. Thus, Mesd and RAP probably bind to different, but perhaps adjacent sites on LRP6. Alternatively, binding of one chaperone may alter the conformation of the receptor such that binding of the other becomes less optimal.

**Mesd antagonizes ligand binding to LRP6**

Wnt proteins and DKK proteins are two important groups of LRP6 extracellular ligands. By using co-immunoprecipitation, several group reported that LRP6 can bind Wnt1, Wnt3a and Xenopus Wnt8 (Tamai et al., 2000; Mao et al., 2001; Kato et al., 2002; Itasaki et al., 2003; Liu et al., 2003). However, the ability of Mesd in modulation DKK1 binding to LRP6 suggests that Mesd may have a physiological role in the regulation of LRP5/6 function at the cell surface. In this regard, it is interesting to note that the LRP5 mutation that causes high bone mass (G171V) disrupts LRP5 interaction with Mesd (Zhang et al., 2004) but allows for autocrine Wnt signaling within the intracellular compartments. We examined total cellular Mesd expression by western blotting in over ten human normal and cancer cell lines and found that Mesd is universally expressed at a similar level in all cell lines tested. However, we were unable to detect Mesd in the concentrated conditioned media of several human cell lines (data not shown). It is possible that regulated secretion of Mesd occurs only under certain physiological or pathophysiological conditions. Alternatively, Mesd may bind to mature LRP5/6 in certain

**Fig. 9.** Mesd inhibits DKK1 binding to LRP6. (A–C) Serum-free conditioned medium was harvested from HEK293 cells transiently transfected with cDNA for human Myc-DKK1 and allowed to bind to LRP6-transduced HT1080 cells (B,C) and control cells (A) in the absence (AB) or presence (C) of 1 μM Mesd. Cell-surface-bound Myc-tagged DKK proteins were fixed and detected by immunofluorescence staining with anti-Myc antibody. (D) DKK1 binding to cell surface LRP6 is inhibited by Mesd. Binding of $^{125}$I-DKK1 (5 nM) to LRP6-transduced HT1080 cells or the control cells was carried out for 3 hours at 4°C in the presence (total) or presence of 500 nM RAP or 500 nM Mesd. Values are the means of triple determinations with the s.d. indicated by error bars. Bar, 10 μm.
intracellular compartments (e.g. endosomes) and regulate autocrine Wnt signaling. Future studies are required to examine these possibilities.

The ability of RAP to universally inhibit ligand binding prompted the use of recombinant RAP as an antagonist in the study of LRP and other members of the LDL receptor family, including examination of in vivo functions of these receptors. Thus, the ability of Mesd to regulate LRP6 ligand binding suggests that recombinant Mesd could also be a useful tool in the study of ligand-LRP6 interactions. Application of recombinant Mesd protein to cells may also allow for detection of LRP6-mediated Wnt signaling that is inhibited by endogenous DKK.

**LRP6 endocytosis**

Traditionally, all members of the LDLR family have been regarded as cell surface endocytosis receptors that function in delivering their ligands to lysosomes for degradation, although recent studies have revealed new important roles for these receptors in signal transduction. A common characteristic of the LDLR family members is that at least one copy of the NPXY sequence is found within the cytoplasmic tails of most members of the LDLR family. For the LDLR, this NPXY motif serves as a signal for receptor endocytosis through coated pits (Chen et al., 1990). For LRP, the YXXL motif, but not the two NPXY sequences, within the cytoplasmic tail of LRP serves as the dominant signal for LRP-mediated endocytosis (Li et al., 2000). LRP belongs to the class of receptors which undergo constitutive endocytosis in the presence or absence of ligand. This feature may be determined by the constant exposure of its endocytosis signals, and is highlighted by its cell surface distribution concentrated within clathrin-coated pits.

The cytoplasmic tails of LRP5 and LRP6 are comprised of 207 and 218 amino acid residues, respectively. Unlike other members of the LDLR family, LRP5 and LRP6 lack the conserved NPXY motif but contain several putative di-leucine and YXXφ (where φ is a bulky hydrophobic amino acid) motifs (Brown et al., 1998; Kim et al., 1998). These motifs may serve as endocytosis signals for LRP5 and LRP6. However, as we demonstrate here, LRP6 does not undergo constitutive endocytosis in the absence of ligand. It has been reported that the single-pass transmembrane protein Kremen1 and Kremen2 play an important role in LRP5/6 degradation (Mao et al., 2002; Davidson et al., 2002; Mao and Niehrs, 2003). Kremen proteins are DKK receptors that regulate Wnt/β-catenin signaling. Upon binding to DKK1, Kremen proteins are recruited into a complex with LRP6, which leads to rapid endocytosis and removal of LRP6 from the plasma membrane. As the Kremen cytoplasmic tail is neither conserved nor required for Kremen-DKK-LRP6 complexes internalization, the function of Kremens may be to allow LRP6 endocytosis by unmasking the putative endocytosis signals in the LRP6 cytoplasmic tail.

Mesd is a special chaperone for LRP5 and LRP6

One of the predominant characteristics of LDLR family members is the presence of multiple classes of conserved modules that include the β-propeller/EGF modules and clusters of cysteine-rich ligand-binding repeats. LRP5/6 has four β-propeller/EGF modules and three ligand-binding repeats (Brown et al., 1998; Kim et al., 1998). The formation of correct disulfide bonds during receptor folding presents a challenging task for ER chaperones. The function of Mesd (as well as RAP) during folding may be primarily to inhibit indiscriminate disulfide bond formation, in particular intermolecularly between different receptor molecules during and after their translation. Specifically, Mesd is required for the maturation of these β-propeller/EGF modules through the secretary pathway (Culi et al., 2004).

The majority of ER chaperones bind only to unfolded, misfolded or folding intermediates of their target proteins. However, RAP remains bound to the LDLR family members to safeguard their trafficking through the early secretory pathway by antagonizing premature ligand binding (Bu et al., 1995; Willnow et al., 1996). Results from our current studies suggest that Mesd may also possess this feature. We showed that Mesd binds to cell surface LRP6 and LRP5, and that Mesd antagonizes DKK1 binding to mature LRP6. The possibility that Mesd escort/safeguards mature LRP5/6 in the secretary and/or endocytic pathways requires further investigation.

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