Low-density-lipoprotein-receptor-related protein (LRP) interacts with a GTP-binding protein

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The low-density-lipoprotein-receptor-related protein (LRP) binds and internalizes numerous ligands, including lipoproteins, proteinase–inhibitor complexes and others. We have shown previously that LRP-mediated ligand internalization is dependent on cAMP-dependent protein kinase (PKA) activity. Here, we investigated whether ligation of LRP increases the intracellular cAMP level and PKA activity via a stimulatory GTP-binding protein. Treatment of LRP-expressing cell lines with the LRP ligands lactoferrin or urokinase-type plasminogen activator caused a significant elevation in cAMP and stimulated PKA activity in a dose-dependent manner. Addition of the 39 kDa receptor-associated protein (RAP), an antagonist for ligand

interactions with LRP, blocked the lactoferrin-induced increase in PKA activity, demonstrating a requirement for ligand binding to LRP. Incubation of cell membrane fractions with lactoferrin increased GTPase activity in a time- and dose-dependent manner, and treatment with LRP ligands suppressed cholera-toxin-mediated ADP-ribosylation of the $G_s\alpha$ subunit of a heterotrimeric G-protein. Affinity precipitation of LRP with RAP resulted in co-precipitation of two isoforms of $G_s\alpha$ from detergent extracts. We thus conclude that LRP is a signalling receptor that associates directly with a stimulatory heterotrimeric G-protein and activates a downstream PKA-dependent pathway.

INTRODUCTION

The low-density lipoprotein (LDL)-receptor-related protein (LRP), also known as the α_2 -macroglobulin receptor, is a multi-ligand endocytosis receptor and is thought to play a critical role in several vascular processes, including lipoprotein metabolism and the regulation of fibrinolytic activity [1–3]. Cell-surface LRP is a heterodimer consisting of a 515 kDa heavy chain, to which ligands bind, and a non-covalently associated 85 kDa light chain, which contains the transmembrane and cytoplasmic domains. As a member of the LDL receptor family, LRP contains the characteristic structural motifs of this family. One such motif, the NPXY sequence, which functions as a signal for clustering and endocytosis of receptors in coated pits, is present in two repeats in the 100-amino-acid cytoplasmic portion of LRP [1]. LRP binds and mediates the internalization of a diverse group of molecules, ranging from lipoproteins (including apolipoprotein E, chylomicron remnants, β -migrating very-low-density lipoprotein and lipoprotein lipase), to proteinases and proteinase–inhibitor complexes [including α_2 -macroglobulin proteinase complexes, the plasminogen activators (PAs) tissue-type PA (tPA) and urokinase-type PA (uPA), as well as PA complexed with inhibitors] to unrelated molecules (such as lactoferrin and thrombospondin) [1–3]. An intracellular protein of approx. 39 kDa, termed receptor-associated protein (RAP), can inhibit the binding of all these ligands to LRP [1–3]. The physiological role of RAP is that of a molecular chaperone required for the proper folding and export of LRP from the endoplasmic reticulum by preventing the premature binding of co-expressed ligands [4].

Receptor-mediated endocytosis involves the sorting of mem-

brane proteins into specialized regions of the plasma membrane and a series of regulated membrane fusion events [5,6]. For several receptors that use the clathrin-coated-pit-mediated endocytosis pathway, internalization depends on factors such as ligand occupancy, phosphorylation state and downstream signal transduction [7,8]. The mechanisms involved in the endocytosis of the members of the LDL receptor family are not completely understood. LRP, like the LDL receptor [9], is believed to move spontaneously into coated pits and enter cells continuously, independent of the presence of ligand. We recently demonstrated that the inhibition of cAMP-dependent kinase (PKA) activity results in suppression of the internalization of LRP ligands [10]. This raises the possibility that LRP ligation induces activation of PKA via a stimulatory heterotrimeric G-protein. Here, we evaluate whether ligation of LRP leads to the activation of a G-protein-dependent signalling pathway. We investigate the effect of LRP ligation on the intracellular cAMP concentration and PKA activity in LRP-expressing cell lines. We further determine whether LRP ligation induces the activation of $G_s\alpha$ and whether it enhances GTPase activity and, finally, whether LRP is found in close physical proximity to $G_s\alpha$.

EXPERIMENTAL

Materials

High-molecular-weight uPA purified from human plasma, polyclonal rabbit anti-(human uPA receptor) antibody #399R, monoclonal anti-(human LRP light chain) antibody #3501 and recombinant human PA inhibitor-type 1 (PAI-1) were purchased from American Diagnostica, Inc. (Greenwich, CT, U.S.A.).

Abbreviations used: CT, cholera toxin; GST, glutathione S-transferase; LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; PA, plasminogen activator; PAI-1, PA inhibitor type 1; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor; RAP, receptor-associated protein; tPA, tissue-type PA; uPA, urokinase-type PA.

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Recombinant human pro-uPA was generously donated by Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL, U.S.A.). Complexes of uPA and PAI-1 were obtained by incubation of high-molecular-weight uPA with a 10-fold molar excess of PAI-1 for 90 min at 20 °C. The polyclonal rabbit anti-(human LRP) antibody #777 has been described [11] and was kindly provided by Dr. Dudley K. Strickland (American Red Cross, Rockville, MD, U.S.A.). Polyclonal rabbit anti-G_sα, anti-G_oα and anti-G_iα antibodies and cholera toxin (CT) were from Calbiochem (San Diego, CA, U.S.A.). The plasmid pGEX-KG-RAP, coding for human RAP as a glutathione S-transferase (GST) fusion protein, was made available by Dr. Joachim Herz (University of Texas, Southwestern Medical Center, Dallas, TX, U.S.A.). GST-RAP was expressed in *Escherichia coli* and purified using the GST purification module from Pharmacia (Piscataway, NJ, U.S.A.).

Cell culture

M21 human melanoma cells [12] were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. HepG2 human hepatocellular carcinoma cells were obtained from the American Type Culture Collection (A.T.C.C. HB-8065) and were grown in Eagle's minimal essential medium containing 10% (v/v) fetal bovine serum. Before testing, monolayers of cells at 70–80% confluency were serum-starved for 24 h.

Co-precipitation and immunoblotting

Monolayers of cells were washed in PBS (0.01 M phosphate/0.15 M NaCl, pH 7.2) and lysed on ice for 10 min with 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 60 mM n-octyl β-D-glucopyranoside and proteinase inhibitor cocktail (Complete®; one tablet for 10 ml of lysis buffer; Boehringer Mannheim, Indianapolis, IN, U.S.A.). Cell lysates were harvested by scraping with a rubber policeman and transferred to an Eppendorf tube. Cellular debris was removed by centrifugation at 1300 g for 15 min. A 1 ml portion of supernatant was incubated with GST-RAP for 2 h at 4 °C, followed by incubation with glutathione-agarose beads overnight at 4 °C. Co-precipitations were collected by centrifugation at 735 g for 5 min at 4 °C. The pellet was washed extensively with PBS, resuspended in non-reducing electrophoresis sample buffer (Novex, San Diego, CA, U.S.A.) and boiled for 3 min. Samples were separated by SDS/PAGE (14% Tris/glycine gel; Novex) and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, U.S.A.). Membranes were blocked with 10% (w/v) milk powder and used for immunoblot analysis with rabbit polyclonal or mouse monoclonal antibodies and appropriate horseradish-peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL, U.S.A.) was used for visualization of labelled proteins.

[³²P]ADP-ribosylation by CT

Cells were homogenized in PBS, pH 7.5, containing 250 mM sucrose and proteinase inhibitor cocktail (Complete®; one tablet for 10 ml of buffer) and centrifuged for 5 min at 735 g; the pellet was discarded. The supernatant was subjected to 30 min of high-speed centrifugation (189000 g; SW 50.1 Beckman rotor) at 4 °C. The pellet obtained after high-speed centrifugation contained a crude cell membrane fraction and was resuspended in 100 μl of ribosylation buffer containing 100 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 100 mM GTP, 1 mM ATP, 10 mM thymidine and proteinase inhibitor cocktail. LRP ligands, polyclonal anti-

LRP antibodies or anti-(uPA receptor) antibodies were added and incubated at 30 °C for 5 min prior to the addition of 2 μg of CT and 50 μCi of [³²P]NAD⁺ (Amersham; 10 mCi/ml). The reaction was then incubated for 30 min at 30 °C. The membranes were collected by centrifugation at 11750 g for 10 min, washed twice in PBS and resuspended in non-reducing electrophoresis sample buffer. The samples were boiled for 3 min and analysed by SDS/PAGE. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane, air-dried and exposed on a phosphor screen cassette (Molecular Dynamics, Sunnyvale, CA, U.S.A.) overnight at room temperature. The phosphor screen was scanned by a PhosphorImager SI® (Molecular Dynamics), and the extent of [³²P]ADP-ribosylation was quantified by the image analysis software of the PhosphorImager SI.

PKA assay

PKA activity was determined by using the PKA assay system from Gibco/BRL (Grand Island, NY, U.S.A.). Briefly, monolayers of cells were incubated with uPA or lactoferrin for 20 min at 37 °C, then rinsed twice with PBS, scraped and homogenized in extraction buffer (Gibco/BRL) containing 1 mM 3-isobutyl-1-methylxanthine and proteinase inhibitor cocktail (Complete). Cellular debris was removed by centrifugation and the supernatant was divided into aliquots in triplicate on a 96-well microtitre plate and incubated for 10 min with cAMP, PKA inhibitor (PKI; Gibco/BRL) or PKI plus cAMP. The PKA substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide; Gibco/BRL) and [γ-³²P]ATP (Amersham; 20 μCi/ml) were added to the reaction, followed by incubation for 10 min at 30 °C. Activated PKA in the reaction mixtures was determined by following the manufacturer's instructions.

Determination of cellular cAMP levels

Monolayers of cells were incubated with uPA or lactoferrin for 15 min at 37 °C and washed twice with PBS. cAMP was extracted with 5 mM acetic acid containing 1 mM 3-isobutyl-1-methylxanthine. After a 10 min incubation on ice, the cells were scraped and transferred to Eppendorf tubes. The samples were centrifuged at 1300 g for 10 min in a microcentrifuge at 4 °C. The supernatants were neutralized and the cAMP concentration was determined by the ¹²⁵I-cAMP scintillation proximity assay (Amersham) according to the manufacturer's instructions. The pellets were solubilized in 0.1 M NaOH and the protein concentration was determined by the BCA (bicinchoninic acid) protein assay method (Pierce, Rockford, IL, U.S.A.). The cAMP levels were normalized to the protein concentration, and expressed as fmol of cAMP/μg of protein.

Determination of GTPase activity

The determination of GTPase activity was carried out as described previously [13], with modifications. Cells were scraped with Versene containing proteinase inhibitor cocktail (Complete) and homogenized with 15 strokes in a glass Dounce homogenizer on ice. The homogenate was passed through a fine-gauge syringe needle and centrifuged for 10 min at 735 g in a microcentrifuge. A crude preparation of cell membranes was collected by ultracentrifugation of the supernatant for 30 min at 189000 g at 4 °C and resuspended in 100 μl of GTPase assay buffer. A 5 μg portion of membrane protein and 0.5 μM [γ-³²P]GTP (Amersham) were incubated at 37 °C in the presence or absence of lactoferrin. The reaction was terminated by the addition of ice-cold PBS containing 5% activated charcoal, unhydrolysed

[γ - 32 P]GTP was removed and aliquots were counted for radioactivity in a scintillation counter.

RESULTS

LRP ligation increases cellular cAMP levels and activates PKA

Previously, we demonstrated that the internalization of LRP ligands, such as uPA, activated α_2 -macroglobulin or lactoferrin, is inhibited in the presence of inhibitors of PKA or when expression of PKA is suppressed with a specific antisense oligonucleotide. These findings prompted us to investigate whether ligation of LRP directly affects PKA activity. Because PKA activity is dependent on intracellular cAMP, we tested the effect of two LRP ligands, uPA and lactoferrin, on intracellular cAMP levels. M21, an LRP-expressing human melanoma cell line [10], was incubated with lactoferrin or uPA for 15 min at 37 °C. Upon treatment of M21 cells with 100 nM uPA, cAMP levels increased from a basal level of 2.0 ± 0.21 fmol of cAMP/ μ g of protein in cell extracts to 12.0 ± 1.24 fmol/ μ g; the cAMP level was 13.5 ± 1.43 fmol/ μ g after treatment with 100 nM lactoferrin. Another LRP-expressing cell line, HepG2, showed similar rises in cAMP levels after stimulation with lactoferrin or uPA.

Next, we evaluated whether LRP ligation increases the activity of PKA. We utilized an assay system that measures the relative phosphorylation of kemptide in the presence and absence of a specific PKA inhibitor, PKI. In this assay, stimulation of M21 cells with 100 nM uPA for 20 min increased the activity of PKA 2-fold, and stimulation of HepG2 cells with 100 nM uPA led to an approx. 2.5-fold increase in PKA activity (Table 1). The effect of different doses of lactoferrin on PKA activity in both cell lines was tested; 200 nM lactoferrin was found to lead to maximal PKA activity (Table 1). To demonstrate that the LRP-ligand-induced increase in PKA activity depended on ligand binding to LRP, we pre-incubated cells with RAP, an LRP chaperone and ligand that has been shown to inhibit binding of all other ligands [4]. When cells were pre-incubated with a 20-fold excess of GST–RAP, lactoferrin did not induce an increase in PKA activity

Table 1 LRP ligands increase PKA activity

PKA activity was determined as described in the Experimental section. Results are means $\pm$ S.E.M. of three independent experiments. Significance of differences compared with respective untreated control (two-tailed Student's *t*-test): **P* < 0.01; ***P* < 0.001; ****P* < 0.0001.

Cells	Treatment	Relative PKA activity (% inducible by cAMP)
M21	None	4.1 ± 0.6
	uPA (100 nM)	$7.1 \pm 0.8^*$
	Lactoferrin (50 nM)	$7.9 \pm 0.9^*$
	Lactoferrin (100 nM)	$12.2 \pm 1.3^{**}$
	Lactoferrin (200 nM)	$32.3 \pm 2.7^{***}$
	Lactoferrin (1 μ M)	$33.8 \pm 2.9^{***}$
HepG2	None	13.4 ± 1.5
	uPA (100 nM)	$30.1 \pm 2.7^{**}$
	Lactoferrin (50 nM)	$32.1 \pm 3.1^{**}$
	Lactoferrin (100 nM)	$34.6 \pm 3.4^{**}$
	Lactoferrin (200 nM)	$36.2 \pm 3.7^{**}$
	Lactoferrin (1 μ M)	$37.0 \pm 5.8^*$
M21	None	4.5 ± 0.8
	Lactoferrin (100 nM)	$12.8 \pm 1.7^*$
	GST–RAP (200 nM)	3.9 ± 0.9
	GST–RAP (2 μ M) + lactoferrin (100 nM)	5.2 ± 0.9

Figure 1 LRP ligation attenuates CT-mediated ADP-ribosylation

Crude membrane fractions of M21 cells were prepared and treated with LRP ligands (5 μ g) as indicated, and CT-dependent ADP-ribosylation was determined, as described in the Experimental section. The extent of [32 P]ADP-ribosylation was visualized and quantified by the image analysis software of the PhosphorImager SI[®]. Aliquots were also blotted and stained with a G_{α} -specific antibody to confirm that ADP-ribosylation was determined in the presence of equal amounts of protein. See Table 2 for quantification of bands.

(Table 1). Note that exposing M21 cells for 15 min to GST–RAP alone did not result in a change of PKA activity (Table 1), suggesting that RAP is not a signalling ligand for LRP.

LRP ligation stimulates the dissociation of heterotrimeric G-proteins and GTPase activity

Stimulatory heterotrimeric G-proteins containing the G_{α} subunit provide a link between the ligation of transmembrane receptors and the activation of PKA. ADP-ribosylating toxins efficiently and irreversibly ribosylate the $\alpha\beta\gamma$ complex of an inactive heterotrimeric G-protein [14,15]. CT specifically modifies G_{α} -containing G-proteins, and therefore we employed CT to evaluate whether LRP ligands affect the activation state of G_{α} . Western blotting with a G_{α} -specific antibody identified the 46 and 52 kDa isoforms of G_{α} in M21 cells (Figure 1, lower panel). Crude membranes from M21 cells were isolated and pretreated with one of the LRP ligands lactoferrin, pro-uPA, uPA or uPA–PAI-1 complexes prior to ADP-ribosylation with CT. Lactoferrin treatment resulted in a marked decrease in CT-mediated [32 P]ADP-ribosylation compared with untreated cell membranes of M21 cells (Figure 1). Similarly, treatment with pro-uPA, uPA or uPA–PAI-1 also led to a decrease in the CT-mediated ADP-ribosylation of G_{α} (Figure 1). Quantification of the decrease in CT-mediated ADP-ribosylation from three independent experiments is shown in Table 2. These data demonstrate that the binding of LRP ligands induces the activation and dissociation of a heterotrimeric G-protein containing the G_{α} subunit. As shown in Figure 1, incubation of crude membrane fractions with anti-LRP polyclonal antibody resulted in a 40–60 % decrease in CT-mediated ADP-ribosylation. The uPA receptor is another cell-surface receptor that binds pro-uPA, uPA and uPA–PAI-1. An anti-(uPA receptor) polyclonal antibody, however, did not significantly decrease CT-mediated ADP-ribosylation, indicating that the uPA receptor is not directly involved in activating the stimulatory G-protein. CT-mediated ADP-ribosylation was also suppressed by LRP ligands in HepG2 cells (results not shown), corroborating our results in M21 cells.

To obtain support for our hypothesis that LRP is coupled to a G-protein, we examined the ability of lactoferrin to stimulate GTPase activity in a crude cell membrane preparation from M21 cells. As demonstrated in Figure 2, lactoferrin stimulated GTPase activity in a dose- and time-dependent manner.

Table 2 Quantification of CT-mediated ADP-ribosylation of $G_{s\alpha}$ in M21 cells

The relative intensity of ^{32}P -labelled $G_{s\alpha}$ in three independent experiments was quantified with ImageQuant® (Molecular Dynamics). Results are means $\pm$ S.E.M.

Treatment	ADP-ribosylation (%)
Untreated	100
Lactoferrin	44 $\pm$ 4.1
Pro-uPA	65 $\pm$ 5.4
uPA	62 $\pm$ 4.9
uPA-PAI-1	60 $\pm$ 4.5
Anti-LRP	41 $\pm$ 3.5
Anti-(uPA receptor)	95 $\pm$ 6.9

Figure 2 LRP stimulates GTPase activity

M21 cells were incubated with 200 nM lactoferrin (broken line) or 1 mM lactoferrin (solid line) for 10, 20 or 30 min at 37 °C. GTPase assays were performed, as described in the Experimental section. Results are from one experiment, representative of three.

LRP is physically associated with $G_{s\alpha}$

We used a co-affinity precipitation approach to test whether LRP and a stimulatory heterotrimeric G-protein are physically associated in the cell membrane. To this end, cell lysates prepared in n-octyl β -D-glucopyranoside were incubated with the high-affinity LRP ligand GST-RAP, followed by precipitation with glutathione-agarose beads, separation by SDS/PAGE and Western blot analysis. LRP consists of a 515 kDa heavy chain and an 85 kDa light chain, and GST-RAP precipitated LRP from cell lysates of M21 and HepG2 cells, as detected by Western blotting with polyclonal anti-LRP antibody #777 and an LRP-light-chain-specific monoclonal antibody (Figure 3A). The LRP heavy chain in M21 cell lysates gave a rather weak signal, possibly due to some breakdown during the affinity precipitation procedure. GST-RAP co-precipitated two oligomers of $G_{s\alpha}$, of molecular masses 52 and 46 kDa (Figure 3B). In contrast, when cell lysates were precipitated with glutathione-agarose beads in the absence of GST-RAP, $G_{s\alpha}$ was not detectable (results not shown).

GST-RAP-precipitated material from M21 cell lysates was also blotted with antibodies directed against the $G_{o\alpha}$ and $G_{i3\alpha}$ subunits. Compared with $G_{s\alpha}$, $G_{o\alpha}$ and $G_{i3\alpha}$ were co-precipitated only in trace amounts (Figure 3B). Western blot analysis of 20 μ g portions of total cell lysate from M21 cells demonstrated that

Figure 3 RAP precipitates LRP and co-precipitates a stimulatory G-protein

Cell lysates were incubated with 10 μ g of GST-RAP for 2 h at 4 °C, followed by incubation with 30 μ l of glutathione-agarose beads overnight at 4 °C. After extensive washing, beads were resuspended in electrophoresis buffer, separated by SDS/PAGE, transferred to nitrocellulose and stained with anti-LRP antibodies (A). Aliquots of GST-RAP precipitates were immunoblotted using polyclonal antibodies against $G_{s\alpha}$, $G_{o\alpha}$ and $G_{i3\alpha}$ (B). Total lysates were immunoblotted to demonstrate that $G_{s\alpha}$, $G_{o\alpha}$ and $G_{i3\alpha}$ are present in similar quantities in M21 cells.

$G_{s\alpha}$, $G_{o\alpha}$ and $G_{i3\alpha}$ are present in similar quantities in M21 cells (Figure 3B). This suggests that RAP precipitates LRP and specifically co-precipitates $G_{s\alpha}$, whereas traces of $G_{o\alpha}$ and $G_{i3\alpha}$ may be contaminants. Together, these data indicate that LRP exists in the cell membrane in close physical association with a heterotrimeric G-protein containing the $G_{s\alpha}$ subunit, forming a functional signal-transducing complex.

DISCUSSION

Previously, we demonstrated that the LRP-mediated internalization of several ligands is dependent on PKA activity [10]. Here we show that lactoferrin binding to LRP enhances GTPase activity, raises intracellular levels of cAMP and stimulates PKA activity in two tumour cell lines. uPA, another LRP ligand, activates the same downstream signalling events, whereas RAP binding to LRP does not increase PKA activity. Furthermore, we demonstrate co-affinity precipitation of LRP with $G_{s\alpha}$ and the ability of LRP ligands to suppress the CT-catalysed ADP-ribosylation of $G_{s\alpha}$. Together, these data suggest a physical and functional interaction of LRP with a stimulatory heterotrimeric G-protein in cell membranes of M21 cells. They also suggest the presence of a signalling pathway in which ligation of LRP induces the dissociation of the $G_{s\alpha}$ subunit from a heterotrimeric G-protein, leading to activation of adenylate cyclase and

a subsequent rise in intracellular cAMP. This, in turn, leads to enhanced PKA activity that contributes to the endocytosis of LRP-bound ligand.

It has been proposed previously that LRP ligands can initiate cellular signal transduction events. Misra et al. [16] have shown that binding of the LRP ligands lactoferrin, *Pseudomonas* exotoxin A and lipoprotein lipase increases intracellular calcium and inositol phosphate concentrations in mouse peritoneal macrophages, and that this process involves a pertussis toxin-sensitive heterotrimeric G-protein. In that experimental system, similar to what we demonstrate here, RAP also appears not to be a signalling ligand [17]. Activated α_2 -macroglobulin, another LRP ligand, also elevates intracellular calcium, inositol phosphates and cAMP [18] by a pathway that involves a CT-sensitive G-protein [17]. The signalling events elicited by activated α_2 -macroglobulin, however, appear to be mediated by a thus far uncharacterized high-affinity α_2 -macroglobulin receptor that is distinct from LRP [19]. Another recent study demonstrated that LDL- and high-density-lipoprotein-induced surfactant secretion by alveolar type II cells, which involves increases in phosphoinositide hydrolysis, intracellular calcium concentration and protein kinase C activity, can be inhibited by pertussis toxin, suggesting the possibility that the LDL receptor also interacts with a heterotrimeric G-protein [20]. Members of the LDL receptor family, including LRP, contain only a single transmembrane domain, and their conformation thus differs from that of the classical heptahelical G-protein-coupled receptors. However, it has been shown previously that single-transmembrane receptors, such as the epidermal growth factor receptor and the insulin-like growth factor II receptor, are coupled to heterotrimeric G-proteins [21–23]. The evidence presented here indicates that LRP is another single-membrane-spanning receptor that is coupled to a heterotrimeric G-protein.

Heterotrimeric G-proteins have been implicated in the regulation of vesicle-mediated transport along both the exocytic and endocytic pathways [24–26], and second messengers and protein kinases downstream of heterotrimeric G-proteins have also been shown to influence various transport processes. For instance, cAMP and PKA activity have been reported to stimulate apical-directed transcytosis and secretion in epithelial cells [27–29]. cAMP was also shown to play a regulatory role in the internalization of CD4 and neural-cell-adhesion-related molecules [30,31]. Inhibition of PKA activity inhibits the internalization of LRP ligands [10] which suggests, together with the findings presented here, that a stimulatory heterotrimeric G-protein regulates receptor-mediated endocytosis via LRP.

Endocytosis is an important mechanism regulating the density of cell-surface receptors, and can occur in a ligand-independent, constitutive manner, as well as in a ligation-dependent way. Receptors that activate a signal transduction pathway have been shown to be internalized at a much higher rate when occupied by ligands [32]. Many G-protein-coupled receptors accumulate in clathrin-coated pits and undergo internalization to intracellular sites within minutes of exposure to ligand [33,34]. The search for the structural basis of the interaction of classical G-protein-coupled receptors with the endocytic machinery has revealed a variety of different structural determinants that influence receptor internalization. An NPX₂Y motif conserved in many G-protein-coupled receptors near the seventh transmembrane domain has been found to be critical for the internalization of the human β_2 -adrenergic receptor [34]. The presence of two NPXY motifs in the cytoplasmic domain of LRP may indicate that at least one NPXY motif remains exposed in the absence of ligand, thus allowing continuous interaction with the endocytic apparatus, whereas ligation of LRP results in conformational changes that

enhance intracellular interactions, leading to more efficient internalization. Whether or not the rate of endocytosis of LRP is increased when LRP is occupied by ligands is beyond the scope of the present investigation.

LRP is thought to be involved as an internalization receptor in a variety of biological processes, including the regulation of proteolytic activity and lipoprotein metabolism. It should be considered, however, that LRP may also have functions other than to remove bound ligand from the cell surface. For example, by participating in a signalling pathway, LRP may regulate the expression of its ligands. In this regard, Hardy et al. [35] demonstrated that, in human colon fibroblasts, specific uptake of tPA by LRP is followed by transcriptional down-regulation of tPA mRNA. Interestingly, the tPA promoter in rat cells has been shown to contain a cAMP response element [36]. Little is known about the regulation of expression of LRP itself, but Coukos et al. [37] reported that cAMP suppresses LRP expression in trophoblast cells. LRP is present in senile plaques in Alzheimer patients, and the amyloid precursor protein, which is bound and internalized by LRP [38], was shown to be associated with a heterotrimeric G-protein [39]. Finally, it was shown recently that apolipoprotein E binding to LRP increases neurite outgrowth in different neuronal cells [40,41], suggesting that ligand binding to LRP can influence aspects of neuronal process development.

In the present study, we provide evidence for the coupling of LRP to a heterotrimeric G-protein and for a signal transduction pathway in which LRP ligation activates G_s and thereby a cAMP/PKA-dependent pathway. We propose that LRP ligation directly initiates cellular signal transduction events, thus enabling LRP to alter the activation state of cells in various physiological and pathophysiological processes.

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