Interaction of a 39 kDa protein with the low-density-lipoprotein-receptor-related protein (LRP) on rat hepatoma cells

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We have recently described a PAI-1-independent pathway of tissue-type plasminogen activator (t-PA) uptake and degradation on the rat MH_1C_1 hepatoma cell line. Further studies have implicated the low-density-lipoprotein-receptor-related protein (LRP) as the mediator of plasminogen-activator inhibitor type-1-independent t-PA endocytosis. The LRP is a multi-functional receptor which is shared by a variety of ligands, including α_2-macroglobulin, apoprotein E-enriched β-very-low-density lipoprotein, t-PA and *Pseudomonas* exotoxin A. In each case, binding of ligand to this receptor can be inhibited by addition of the 39 kDa LRP-receptor-associated protein. This protein, which co-purifies with the LRP receptor, is the focus of our present study. ^{125}I-labelled 39 kDa protein binds specifically and with high affinity to a single kinetic binding species on the rat MH_1C_1 cell surface. Scatchard analysis reveals an equilibrium dissociation constant (K_d) of 3.3 ± 0.9 (S.D.) nM, with 380000 ± 190000 (S.D.) binding sites per cell. Cross-linking studies indicate that the specific interaction between MH_1C_1 cells and the 39 kDa protein is mediated by an association with the LRP receptor. The 39 kDa protein strongly inhibits binding of ^{125}I-t-PA, with an apparent K_i value of 0.5 nM. In addition, both unlabelled t-PA and ^{125}I-labelled 39 kDa protein can be co-bound and cross-linked to the same cell-associated LRP receptor. Endocytosis of cell-surface-associated 39 kDa protein was shown to be rapid, with internalized ligand subsequently degraded and released to the extracellular milieu. The rate of uptake and degradation of ^{125}I-labelled 39 kDa protein at 37 °C was determined to be 52 fmol/min per 10^6 cells, and supports a model for active recycling of the LRP receptor.

INTRODUCTION

The low-density-lipoprotein (LDL)-receptor-related protein (LRP) was initially identified by its structural homology to the LDL receptor. Studies to date have suggested that this large cell-surface protein is a multi-functional receptor which participates in endocytosis, and which has the ability to interact with several structurally distinct ligands [1–8]. Recent studies from our laboratory [6] and by Orth et al. [9] demonstrate that LRP is also an hepatic receptor for tissue-type plasminogen activator (t-PA), a serine protease which catalyses the initial and rate-limiting step in fibrinolysis.

The ability of LRP to bind several structurally distinct ligands may relate to its large molecular size and multiple repeated domains. Native LRP is synthesized as a single-chain polypeptide of 600 kDa and thereafter cleaved into two subunits of approx. 520 kDa and 85 kDa. Both subunits remain associated with one another during trafficking to the cell surface [10]. The 520 kDa subunit remains membrane-associated via its interaction with the membrane-spanning 85 kDa subunit. The 31 cysteine-rich repeats arranged as four clusters within the 520 kDa subunit are presumably responsible for ligand binding to this receptor.

LRP has been purified from human placenta [1] and from rat liver [3]. In addition to the 520 kDa and the 85 kDa subunits, these preparations contained a polypeptide referred to as the 39 kDa protein [1,11] or 40 kDa protein [3,12]. This 39 kDa protein directly binds to LRP with high affinity [11–13]. cDNA cloning and sequencing revealed that the human 39 kDa protein is the homologue of a rat protein previously identified in kidney as a component of the Heymann nephritis antigen gp330 [14] and is also the equivalent of the mouse heparin-binding protein 44 [15].

The function of this 39 kDa protein is not yet clear. However, recent studies have shown that binding of the 39 kDa protein to LRP results in the inhibition of receptor-mediated binding and uptake of three of LRP's established ligands: apoprotein E (apo E)-enriched β-very-low-density lipoprotein (β-VLDL) [16], activated α_2-macroglobulin [16] and *Pseudomonas* exotoxin A [8]. This suggests a potential regulatory role for the 39 kDa protein in ligand binding to LRP. In the present study we demonstrate that recombinant 39 kDa protein can specifically bind with high affinity to LRP on rat hepatoma cells, and that binding of this protein to LRP inhibits specific t-PA binding. Chemical cross-linking studies demonstrated a direct interaction between this 39 kDa protein and LRP on the hepatic cell surface. Furthermore, rapid uptake and degradation of the 39 kDa protein by rat hepatoma cells suggests that the level of this protein can be highly regulated *in vivo*.

MATERIALS AND METHODS

Materials

The plasmid *pGEX*-39 kDA was given by Dr. Joachim Herz of the Southwestern Medical Center, University of Texas, Dallas, TX, U.S.A. Activated human thrombin was given by Dr. George Brooze of Washington University, St. Louis. Recombinant single-chain human t-PA expressed in Chinese-hamster ovary cells was provided by Genentech (lot 9124AX). Carrier-free

Abbreviations used: LRP, low-density-lipoprotein-receptor-related protein; apo E, apoprotein E; β-VLDL, β-very-low-density lipoprotein; LDL, low-density lipoprotein; t-PA, tissue-type plasminogen activator; DTSSP, dithiobis(sulphosuccinimidylpropionate); PAI-1, plasminogen-activator inhibitor type 1; PMSF, phenylmethanesulphonyl fluoride; TCA, trichloroacetic acid.

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$Na^{125}I$ was purchased from DuPont–New England Nuclear Products. [^{35}S]Methionine and Hyperfilm-MP autoradiography film were purchased from Amersham Corp. Freund's adjuvant and complete Eagle's minimum essential medium with Earle's salts were purchased from Gibco BRL, and medium lacking L-methionine was obtained from the Washington University Center for Basic Cancer Research. Agarose-immobilized-glutathione beads and GSH were obtained from Sigma Chemical Co. Dithiobis(sulphosuccinimidylpropionate) (DTSSP) and Iodogen were purchased from Pierce Chemical Co. Protein-A beads were obtained from Repligen. Pronase was purchased from Calbiochem. All other chemicals were of analytical-reagent quality.

Antibodies

High-affinity polyclonal antibodies were prepared in New Zealand white rabbits after injection of purified recombinant 39 kDa protein, purified recombinant t-PA or purified LRP in Freund's adjuvant as described previously [17]. Affinity-purified rabbit anti-(human LRP) antibody was provided by Dr. Dudley K. Strickland of the American Red Cross, Rockville, MD, U.S.A.

Radiolabelling of purified proteins

Recombinant 39 kDa protein and t-PA were radiolabelled with carrier-free $Na^{125}I$ by using the Iodogen procedure as described previously [18]. Specific radioactivities for each radiolabelled protein were $(1–2) \times 10^7$ c.p.m./μg of protein as measured by γ-radiation scintillation spectrometry.

Purification of the 39 kDa protein

The procedure for purification of the 39 kDa protein from strains of *Escherichia coli* carrying the over-expression plasmid *pGEX-39 kDa* has been outlined previously [16]. Our procedure, which differs slightly from the above protocol, is described below. Cultures of *E. coli* strain DH5α carrying the over-expression plasmid *pGEX-39 kDa* were grown to mid-exponential phase in LB medium with 100 μg/ml ampicillin at 37 °C. Cultures were cooled to 30 °C and supplemented with 0.01% isopropylthio-β-D-galactoside to induce expression of the glutathione *S*-transferase–39 kDa fusion protein. After induction for 4–6 h at 30 °C, cultures were cooled on ice and collected by centrifugation. Cell pellets were lysed in PBSa with 1% Triton X-100, 1 μM pepstatin, 2.5 μg/ml leupeptin, 0.2 mM phenylmethanesulphonyl fluoride (PMSF) and 1 μM EDTA. This lysate was sonicated with a Branson model 450 Sonifier, and the resulting membranes and other cellular debris were collected by centrifugation at 15000 ***g*** for 15 min. The supernatant from this step was incubated overnight with agarose-immobilized glutathione in PBSa and 0.1% NaN_3. The beads were then washed, and the fusion protein was eluted by competition with 5 mM GSH. After dialysis, the fusion protein was cleaved by an overnight incubation with 100 ng of activated human thrombin per 50 μg of fusion protein. The glutathione *S*-transferase epitope was subsequently removed by further incubation with agarose-immobilized glutathione. All the steps after induction were carried out at 4 °C.

Cell culture and media

Rat MH_1C_1 hepatoma cells were grown in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in humidified air containing 5% CO_2 [18]. Cell monolayers were used at approx. 90% confluence, and the media was replaced the day before each experiment.

Saturation-binding analysis

Cells were grown in 12-well dishes to approx. 10^6 cells per well. Monolayers were taken directly from 37 °C and cooled on ice. The binding buffer used for the recombinant 39 kDa protein was PBSc (PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Each cell monolayer was washed three times with PBSc before addition of binding buffer containing various concentrations of ^{125}I-labelled 39 kDa protein, in either the absence or the presence of an excess of unlabelled protein. After a 90 min incubation, cell monolayers were washed three more times with PBSc and lysed in 'low SDS lysis buffer' (0.0625 M Tris/HCl, pH 6.8, with 0.2% SDS, 10% glycerol and 0.01% Bromophenol Blue) [18]. The amount of cell-associated ligand was determined by γ scintillation spectrometry. In cases where t-PA and the 39 kDa protein were co-bound, the t-PA-binding buffer (PBSa containing 0.2 mM $CaCl_2$ and 10 mM ϵ-amino-n-hexanoic acid) was used [18].

Metabolic labelling

Cells were grown in 10 cm dishes as described above. Cell monolayers were washed three times with pre-warmed Eagle's minimum essential medium (MEM) with Earle's salts lacking L-methionine. After two 15 min preincubations at 37 °C with this same medium, cells were labelled by addition of methionine-deficient MEM containing 0.4 mCi/ml [^{35}S]methionine. Labelling was carried out for 4–5 h at 37 °C. After metabolic labelling, cell monolayers were cooled on ice, washed three times with cold PBSc, and bound with recombinant 39 kDa protein in preparation for chemical cross-linking.

Chemical cross-linking

Saturation binding was performed as described above, but with 10 cm dishes of MH_1C_1 cells. Either unlabelled or [^{35}S]methionine-labelled cells were used. After incubation with radiolabelled and unlabelled ligands, cell monolayers were washed three times with PBSc and incubated with either PBSc alone or PBSc containing 0.5–1 mM DTSSP. After 1 h, cross-linking reactions were quenched by three washes with Tris-buffered saline, and cell monolayers were solubilized in PBSa containing 1% Triton X-100 and 1 mM PMSF. Immunoprecipitations were performed as described below.

Immunoprecipitation

Samples of cell lysates from cross-linking and metabolic-labelling experiments were added to equal volumes of PBSc containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 0.5% BSA and 1 mM PMSF ('immunomix'). When metabolically labelled cells were used, lysates were first pre-cleared by an overnight incubation with rabbit pre-immune serum and a subsequent incubation with an excess of Protein-A beads. Samples were then immunoprecipitated with 0.6 μg of affinity-purified α-39 kDa antibody or affinity-purified anti-(human LRP) antibody. When unlabelled cells were cross-linked to a single radiolabelled ligand, no pre-immune clearance of cell lysates was necessary, and either anti-(rat LRP) immune serum or anti-(30 kDa protein) immune serum was substituted for the affinity-

purified antibody. In some cases, samples of cell lysates were also immunoprecipitated with 30 ml of immune anti-t-PA serum. After each immunoprecipitation, bead samples were divided in half and boiled in 0.0625 M Tris/HCl (pH 6.8)/2% SDS/10% glycerol [19] in either the absence or presence of 5% 2-mercaptoethanol. Samples were then separated by SDS/PAGE, and the gels were subsequently fixed and vacuum-dried. Autoradiography was performed at −70 °C with Hyperfilm-MP autoradiography film.

Endocytosis

Endocytosis experiments were performed as described by Ciechanover et al. [20] and Owensby et al. [21]. Briefly, 6-well dishes containing 10^6 cells/well were preincubated at 4 °C with 14 nM ^{125}I-labelled 39 kDa protein to allow binding. After three washes with PBSc (4 °C) to remove non-specifically associated ligand, each dish was placed at 37 °C, and a pre-warmed solution of 200 nM unlabelled 39 kDa protein was added to initiate internalization. At each time point, one of the dishes was cooled directly on ice, and the overlying medium was removed. Precipitation of the overlying medium was carried out by addition of BSA to 10 mg/ml and trichloroacetic acid (TCA) to 20%. Both the TCA-soluble and TCA-insoluble fractions were counted for radioactivity by γ scintillation spectrometry. In addition, cell monolayers were washed three times with PBSc and incubated for 30 min at 4 °C with a solution of PBSc containing 0.25% Pronase. Cells were subsequently removed from each well and pelleted by centrifugation at 12000 *g* for 1 min. Both the cell pellets and the supernatants after Pronase treatment were counted for radioactivity by γ scintillation spectrometry.

Rate of 39 kDa-protein uptake

This was determined as described previously [22]. Briefly, dishes containing 10^6 cells/well were cooled on ice and washed three times with cold PBSc. With the addition of prewarmed (37 °C) binding buffer containing radiolabelled ligand, in either the absence or the presence of an excess of unlabelled 39 kDa protein, each dish was incubated at 37 °C for a period of between 0 and 120 min. At each time point, one of the dishes was cooled directly on ice, and the overlying medium was removed. Cell monolayers were washed three times with PBSc and lysed in low-SDS lysis buffer for the determination of cell-associated radioactivity. Each sample of overlying medium was precipitated with TCA, and the TCA-soluble fraction was counted for radioactivity by γ scintillation spectrometry.

RESULTS

Specific binding of the 39 kDa protein to the MH_1C_1 cell surface

In order to demonstrate a specific interaction between the 39 kDa protein and rat MH_1C_1 hepatoma cells, saturation-binding analyses were performed. As shown in Figure 1(a), non-specific binding of ^{125}I-labelled 39 kDa protein increased linearly over the range of concentrations tested, whereas total binding increased in a curvilinear fashion, approaching an asymptote above 12 nM. Scatchard analysis [23] (inset to Figure 1a) is consistent with a single homogeneous population of binding sites and yields an equilibrium dissociation constant (K_d) of 3.2 nM with 220000 binding sites per cell, for this particular experiment. The results from ten independent binding experiments indicate a mean K_d value of 3.3±0.9 (S.D.) nM for ^{125}I-labelled 39 kDA protein binding to MH_1C_1 cells, with an average of 380000±190000 (S.D.) binding sites per cell (Table 1a). These data indicate both specific and saturable binding of the 39 kDa protein to a single kinetic species of high-affinity receptor on the MH_1C_1 cell surface.

To investigate the nature of the 39 kDa-protein binding species, cell lysates from each binding experiment were studied by SDS/PAGE (Figure 1b), in which the 39 kDa protein appeared to bind in isolation, without apparent association with any auxiliary binding proteins. We therefore conclude that there is a specific and saturable interaction between the 39 kDa protein and an MH_1C_1 cell-surface molecule(s).

Cross-linking of ^{125}I-labelled 39 kDa protein to the MH_1C_1 cell surface

The 39 kDa protein was originally isolated via its ability to co-purify with LRP [1,2]. To determine if LRP functions as an MH_1C_1-cell-specific 39 kDA-protein receptor, cross-linking

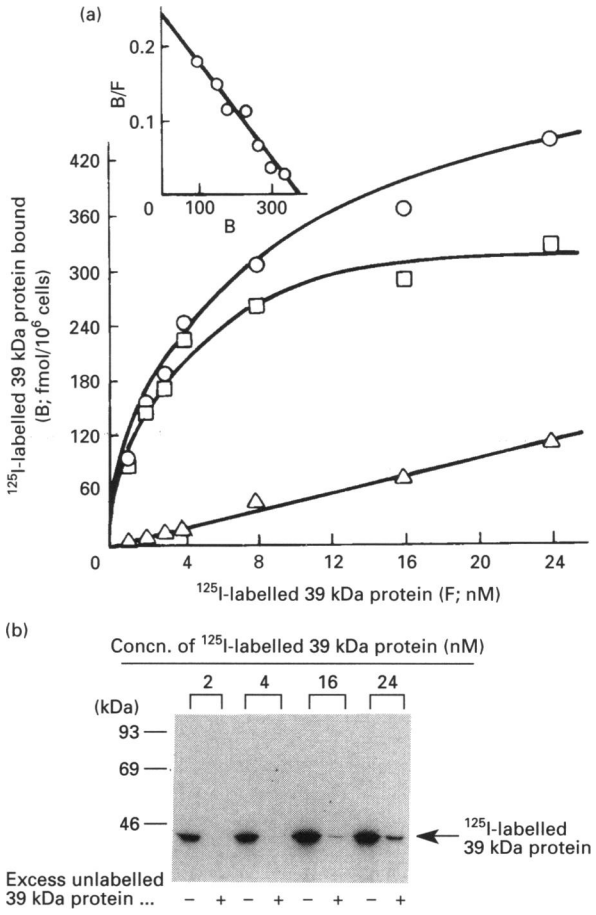

Figure 1 Saturation binding of ^{125}I-labelled 39 kDa protein to MH_1C_1 cells

MH_1C_1 cells were incubated for 1.5 h at 4 °C with increasing concentrations of ^{125}I-labelled 39 kDa protein in the absence or presence of 0.5 μM unlabelled 39 kDa protein. (**a**) Total (○) and non-specific (△) ligand binding was determined. Specific binding (□) was derived as the difference between total and non-specific binding. Each symbol represents the mean of triplicate determinations. Inset: Scatchard plot of specific binding: B, bound, and F, free, ^{125}I-labelled 39 kDa protein. (**b**) Equivalent volumes of post-binding cell lysates, each from about 60000 cells, at selected concentrations were analysed by SDS/PAGE (10% acrylamide, non-reduced), and the gel was exposed to film for 2 days before developing. The position of ^{125}I-labelled 39 kDa protein is indicated. Similar results were seen in 10 independent experiments.

Table 1 Specific binding of ^{125}I-labelled 39 kDa protein and ^{125}I-t-PA to MH_1C_1 Cells

Saturation-binding analyses were performed on MH_1C_1 cells as described in the Materials and methods section, and in the legend to Figure 1. (**a**) represents the mean values (±S.D.) for binding of both the 39 kDa protein and t-PA to MH_1C_1 cells. The cumulative data from all independent binding experiments were used. (**b**) represents the mean values (±S.D.) from only simultaneously performed binding experiments. (**c**) represents data derived from simultaneous binding experiments performed with either untreated MH_1C_1 cells or cells preincubated for 30 min with 0.1% saponin at 4 °C.

Ligand	Preincubation	$10^{-1} \times B_{max.}$ (sites/cell)	K_d (nM)	No. of experiments (n)
(a) Summary				
^{125}I-labelled 39 kDa	None	380±190	3.3±0.9	10
^{125}I-t-PA	None	78±35	4.9±1.3	7
(b) Simultaneous assays				
^{125}I-labelled 39 kDa	None	390±150	3.5±0.9	4
^{125}I-t-PA	None	60±35	5.6±1.5	4
(c) Simultaneous assays performed in the presence or absence of saponin				
^{125}I-labelled 39 kDa	None	250±8	2.7±1.3	3
^{125}I-labelled 39 kDa	Saponin	515±24	9.1±3.6	3

studies were performed. Figure 2 shows the results from a typical experiment. Under conditions where no cross-linker is used (Figures 2c and 2d), a single band corresponding to the 39 kDa protein (arrowhead) is immunoprecipitated only with anti-(39 kDa protein) serum. Samples electrophoresed under either reducing or non-reducing conditions show that the anti-LRP antibody is unable to immunoprecipitate the radiolabelled 39 kDa ligand in the absence of cross-linker. Since there is a marked decrease in the amount of ^{125}I-labelled 39 kDa protein bound in the presence of an excess of unlabelled protein, we conclude that the association of ligand with its cell-surface receptor is specific. In Figures 2(a) and 2(b), the radiolabelled 39 kDa protein is both bound and cross-linked to the MH_1C_1 cell surface. When samples from each immunoprecipitation are analysed under non-reducing conditions (Figure 2a), the complex of ligand-associated radioactivity is immunoprecipitable with both anti-LRP and anti-(39 kDa protein) antibodies. This complex was of very high apparent molecular mass, remaining largely in the stacking gel during electrophoresis (arrows). Again, this interaction was shown to be specific, as the association of ^{125}I-labelled 39 kDa with this high-molecular-mass complex was abrogated in the presence of an excess of unlabelled 39 kDa protein. When these same samples were electrophoresed in the presence of 5% 2-mercaptoethanol to dissociate the cross-linker (Figure 2b), a single 39 kDa binding species was seen. We therefore conclude that there is a specific interaction between the

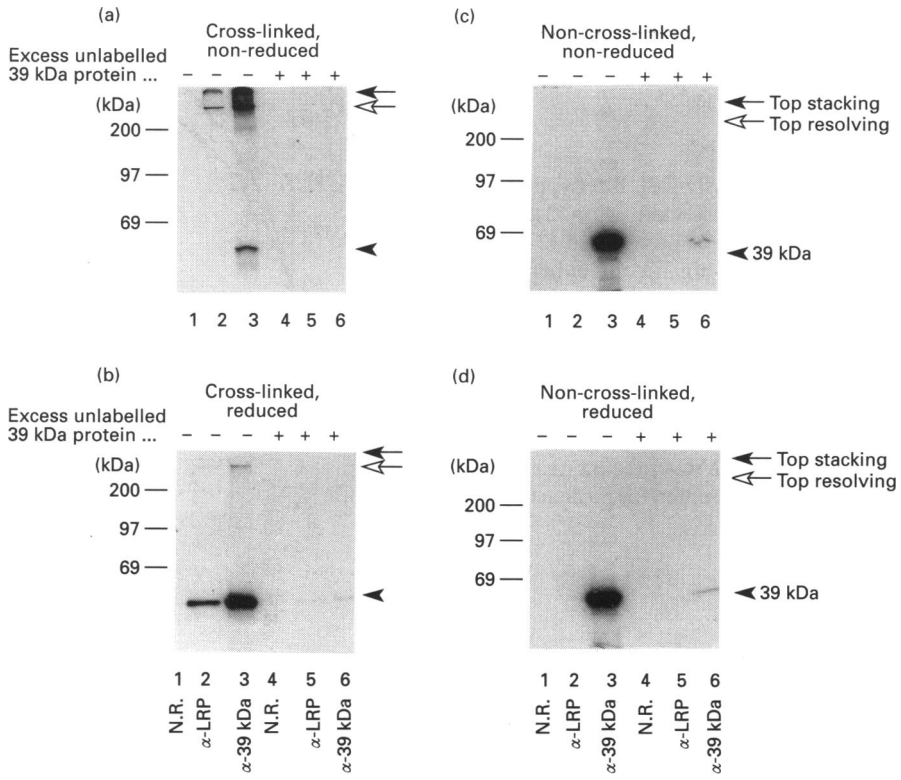

Figure 2 Specific cross-linking of ^{125}I-labelled 39 kDa protein to LRP

Unlabelled MH_1C_1 cells were incubated for 1.5 h with 14 nM ^{125}I-labelled 39 kDa protein in both the presence and the absence of 0.5 μM unlabelled 39 kDa protein. After several washes to remove non-specifically associated ligand, cells were incubated in both the presence (**a** and **b**) and absence (**c** and **d**) of 0.5 mM DTSSP for 1 hour. Cells were then lysed and immunoprecipitated with normal rabbit serum (N.R.; lanes 1 and 4), anti-LRP serum (α-LRP; lanes 2 and 5), or anti-(39 kDa protein) serum (α-39 kDa; lanes 3 and 6) as described in the Materials and methods section. Each immunoprecipitation was analysed by SDS/PAGE (8.5% acrylamide) under both reducing (**b** and **d**) and non-reducing (**a** and **c**) conditions. Gels from samples incubated in the presence of cross-linker were autoradiographed for 3 days at −70 °C, whereas gels incubated in the absence of cross-linker were autoradiographed for only 2 days. The position of ^{125}I-labelled 39 kDa protein is indicated (arrowhead).

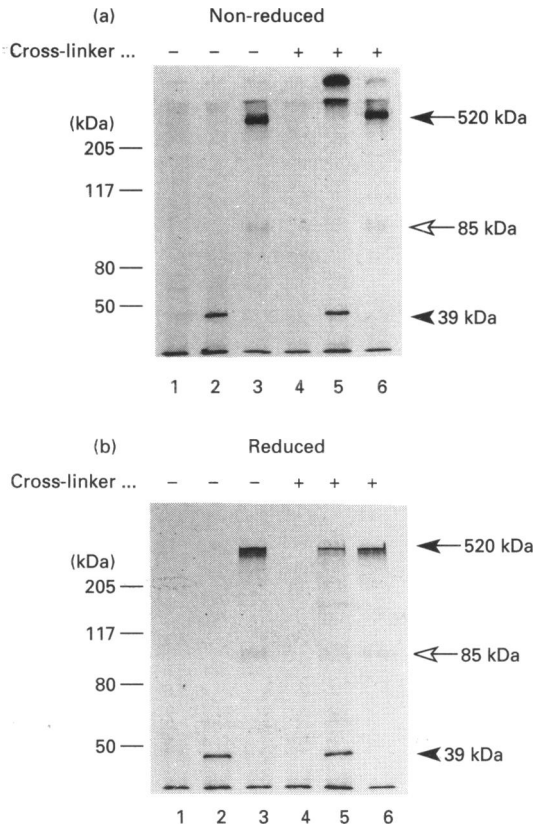

Figure 3 Cross-linking unlabelled 39 kDa protein to the cell surface of metabolically labelled rat MH_1C_1 cells

MH_1C_1 cells were incubated for 5 h at 37 °C with 0.4 mCi/ml-[^{35}S]methionine-supplemented media. After metabolic labelling, cell monolayers were cooled on ice and incubated with a solution of 14 nM unlabelled 39 kDa protein for 1.5 h. At the end of this time, cell monolayers were washed to remove non-specifically associated ligand, and either incubated for 1 h with 0.5 mM DTSSP (lanes 4–6) or in PBSc alone (lanes 1–3). Cells were then lysed and immunoprecipitated with normal rabbit serum (lanes 1 and 4), affinity-purified anti-(rat 39 kDa protein) (lanes 2 and 5), or affinity-purified anti-(human LRP) (lanes 3 and 6). Each immunoprecipitation was then analysed by SDS/PAGE (8.5% acrylamide) under both reducing (**b**) and non-reducing (**a**) conditions. Autoradiography was carried out overnight at −70 °C. The arrowhead indicates the position of the 39 kDa protein. The white and black arrows indicate the position of the 85 kDa and 520 kDa subunits of LRP respectively.

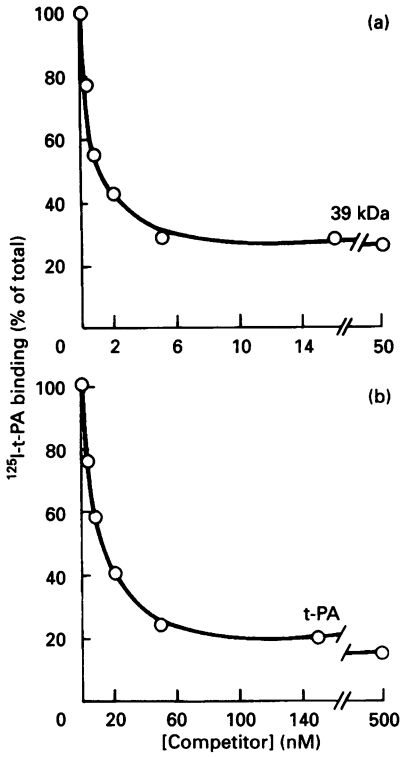

Figure 4 Inhibition of ^{125}I-t-PA binding by rat 39 kDa protein

MH_1C_1 cells were incubated with 4 nM ^{125}I-t-PA in the presence of increasing concentrations of either unlabelled t-PA or 39 kDa protein at 4 °C for 1.5 h. After several washes to remove non-specifically associated radioactivity, cell monolayers were lysed, and the amount of cell-associated radioactivity was determined. Each point represents the mean of triplicate determinations. (**a**) Inhibition of ^{125}I-t-PA binding by the 39 kDa protein. (**b**) Inhibition of ^{125}I-t-PA binding by unlabelled t-PA. Similar results were seen in two independent experiments.

39 kDa protein and the MH_1C_1 cell surface which is mediated, at least in part, by an association with LRP.

The 39 kDa protein is cross-linked predominantly to LRP on the MH_1C_1 cell surface

To determine if LRP is the predominant 39 kDa-protein-binding species, unlabelled 39 kDa protein was cross-linked to the surface of radiolabelled MH_1C_1 cells. Figure 3 shows the results from a typical experiment. When radiolabelled MH_1C_1 cells are cross-linked to unlabelled 39 kDa protein, a complex of very high apparent molecular mass results, which is immunoprecipitable with both the anti-(39 kDa protein) and, to a lesser degree, the anti-(human LRP) affinity-purified antibodies (Figure 3a, lanes 4–6). We have used antibodies to human LRP, as no anti-(rat LRP) affinity-purified antibody is at present available. This high-molecular-mass complex is electrophoretically identical with that seen when ^{125}I-labelled 39 kDa protein is cross-linked to unlabelled MH_1C_1 cells (compare with Figure 2a, lanes 2 and 3). In addition to the cross-linked complex, uncross-linked endogenously labelled 39 kDa protein (lane 5, arrowhead), and both the 520 kDa (black arrow) and 85 kDa (white arrow) subunits of the LRP receptor are visible. When these same immunoprecipitates are electrophoresed in the presence of the reducing agent 2-mercaptoethanol, the cross-linker is cleaved, and the cross-linked complex is dissociated into its component peptides (Figure 3b, lanes 4–6). In both cases, the cross-linked complex is shown to contain almost exclusively the 520 kDa subunit, and to a lesser degree, the 85 kDa subunit of the LRP receptor. In lanes resulting from immunoprecipitation with the anti-(human LRP) antibody, less of the high-molecular-mass cross-linked complex is evident. We reason that this occurs partly because the recognition epitopes for the anti-LRP antibody are obscured by binding of the 39 kDa protein.

When metabolically labelled cell lysates which have been bound with, but not cross-linked to, unlabelled 39 kDa protein are immunoprecipitated with the anti-(39 kDa protein) antibody, only endogenously labelled 39 kDa protein is seen (arrowhead). No high-molecular-mass complex is evident. Also, in the absence of cross-linker, immunoprecipitation of metabolically labelled cell lysates with anti-(human LRP) antibodies results in only the 520 kDa (black arrow) and the 85 kDa (white arrow) subunits of the LRP receptor. Again, no high-molecular-mass complex is evident. Samples electrophoresed under both reducing and non-reducing conditions gave similar results. We therefore conclude

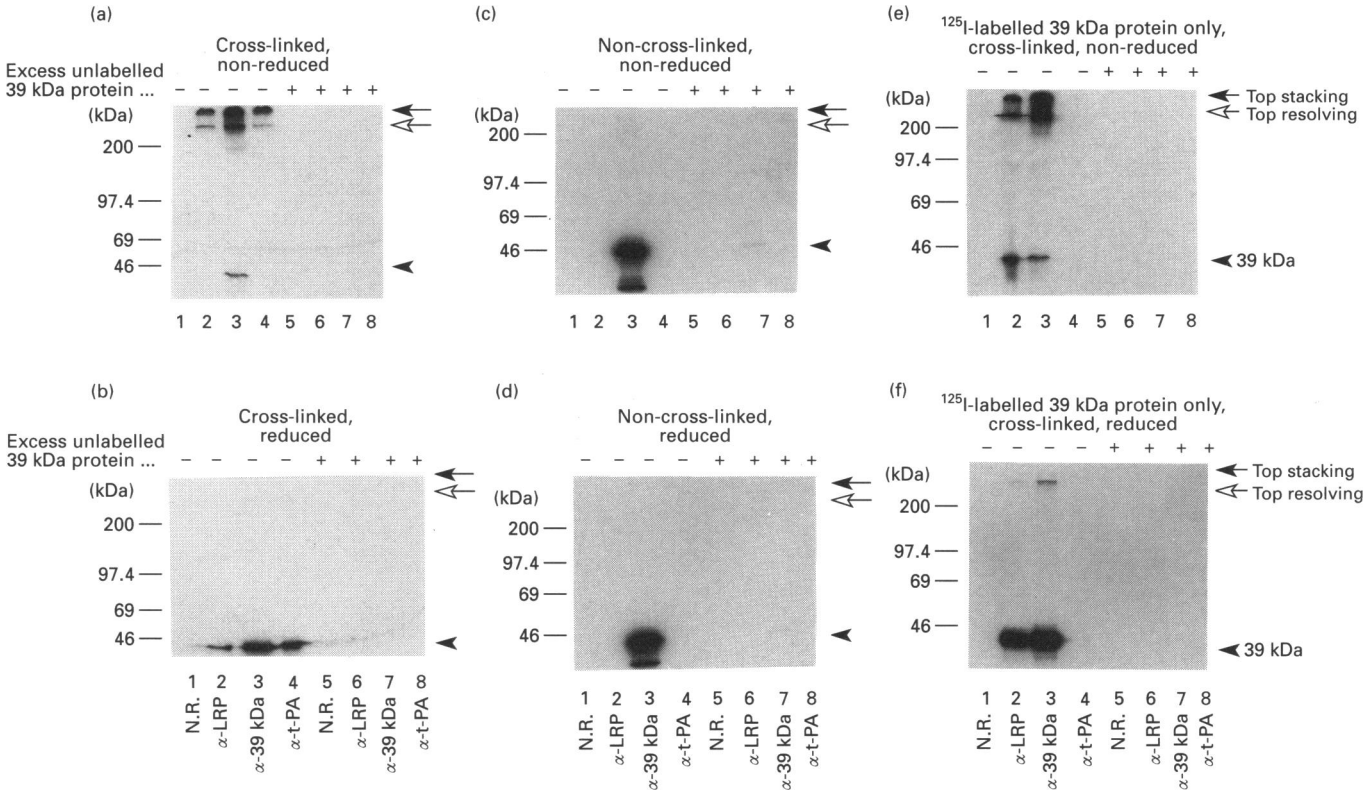

Figure 5 Co-binding and cross-linking of ^{125}I-labelled 39 kDa protein and unlabelled t-PA to rat MH_1C_1 cells

Pre-cooled monolayers of MH_1C_1 cells were incubated at 4 °C with 0.5 nM ^{125}I-labelled 39 kDa protein and 20 nM unlabelled t-PA for 1.5 h, in either the presence or the absence of 0.5 μM unlabelled 39 kDa protein. After several washes to remove non-specifically associated ligand, cells were either left on ice or cross-linked by an additional incubation with 1 mM DTSSP for 1 h at 4 °C. Cells were then lysed and immunoprecipitated as described in the Materials and methods section. Samples were electrophoresed through 8.5% acrylamide under both reducing and non-reducing conditions. Autoradiography was performed at −70 °C for 12 days. For abbreviations see Figure 2 legend.

that the LRP is the predominant 39 kDa-protein-binding species on the MH_1C_1 cell surface.

The rat 39 kDa protein can efficiently inhibit t-PA binding to MH_1C_1 cells

A number of distinct ligands, including α_2-macroglobulin [1–4], apo E-enriched β-VLDL [5,7], t-PA [6] and the 39 kDa protein (the present work) have been shown to bind LRP specifically. Furthermore, both binding and endocytosis of α_2-macroglobulin [16] and apo E-enriched β-VLDL [16] are inhibited in the presence of the 39 kDa protein. We have previously shown that a fusion between the glutathione *S*-transferase C-terminus and the rat 39 kDa protein can inhibit binding of ^{125}I-t-PA to the rat MH_1C_1 cell surface [6]. We have sought here to define more specifically the kinetics of inhibition of t-PA binding to MH_1C_1 cells by the native rat 39 kDa protein. As demonstrated above, ^{125}I-t-PA binds specifically and with high affinity to the MH_1C_1 cell surface [$K_d = 4.9 \pm 1.3$ nM (S.D.); $B_{max.} = 78000 \pm 35000$ (S.D.)] (Table 1a) [18]. Figure 4 shows the results of ^{125}I-t-PA-binding analyses performed in the presence of varying concentrations of either unlabelled t-PA or recombinant rat 39 kDa protein as competitors. Unlabelled t-PA inhibits binding of ^{125}I-t-PA, with an apparent K_i value of 8 nM (Figure 4b). The 39 kDa protein, on the other hand, competes at a much lower concentration, with an apparent K_i value of 0.5 nM. We therefore conclude that the 39 kDa protein is a strong competitor for ^{125}I-t-PA binding to LRP on the MH_1C_1 cell surface, and we routinely test our preparations of 39 kDa protein for this ability.

t-PA and the 39 kDa protein can be cross-linked to the same LRP receptor complex

Since both the 39 kDa protein and t-PA can specifically bind to LRP, and since the 39 kDa protein can inhibit t-PA binding on MH_1C_1 cells, we sought to determine if both ligands could be co-bound to a single LRP molecule on the MH_1C_1 cell surface. Figure 5 shows the results from a typical experiment. When the ^{125}I-labelled 39 kDa protein and unlabelled t-PA are co-bound to the MH_1C_1 cell surface and incubated in the absence of cross-linker (Figures 5c and 5d), the radiolabelled 39 kDa protein is immunoprecipitated from cell lysates with only the anti-(39 kDa protein) antibody (arrowhead). Neither the anti-LRP antibody nor the anti-t-PA antibody show any affinity for the radiolabelled 39 kDa ligand. As before, interaction of the 39 kDa protein with the MH_1C_1 cell surface was shown to be specific by abrogation with an excess of unlabelled 39 kDa protein. When cells were pre-bound with ^{125}I-labelled 39 kDa protein and unlabelled t-PA, and incubated in the presence of cross-linker (Figures 5a and 5b), a high-molecular-mass complex of ligand-associated radioactivity resulted, which was immunoprecipitable with anti-(39 kDa protein), anti-t-PA and anti-LRP antibodies. Each of these interactions was specific, as each was abolished in samples incubated in the presence of an excess of unlabelled 39 kDa

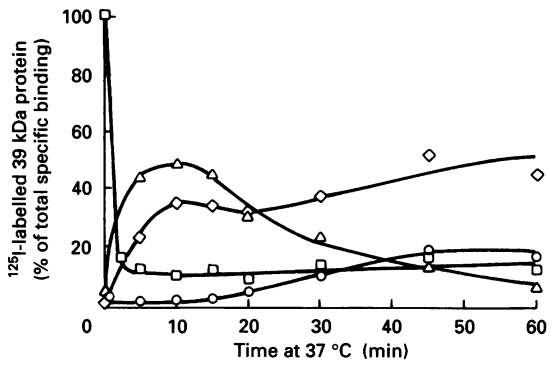

Figure 6 Rapid endocytosis and degradation of surface-bound ^{125}I-labelled 39 kDa protein by MH_1C_1 cells

MH_1C_1 cells were incubated with 14 nM ^{125}I-labelled 39 kDa protein in both the presence and the absence of 0.5 μM unlabelled 39 kDa protein at 4 °C for 1.5 h. After several washes to remove non-specifically associated radioactivity, cells were incubated at 37 °C in the presence of 200 nM unlabelled 39 kDa protein. At each time point, cell monolayers were chilled rapidly on ice. The overlying medium was removed and precipitated in the presence of 10 mg/ml BSA and 20% TCA. Both TCA-soluble radioactivity (○; degraded ligand) and total radioactivity (◇; both dissociated and degraded ligand) were determined. Cell monolayers were treated with Pronase, and the amounts of both Pronase-sensitive (□; plasma-membrane-associated) and Pronase-resistant (△; cell-associated) ligand were determined. Each data point represents specific radioactivity (i.e. the difference of total and non-specific) and is the mean of triplicate determinations. Similar results were seen in three independent experiments.

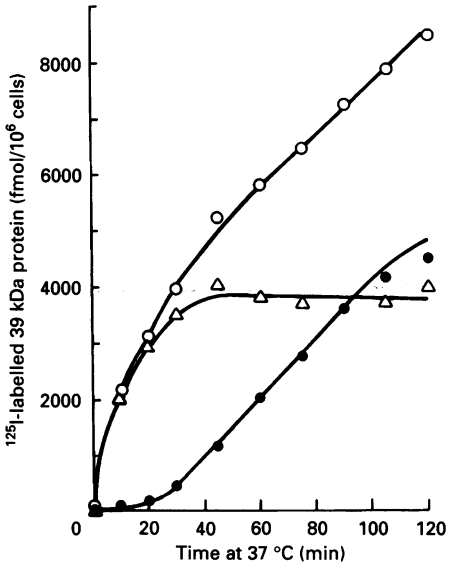

Figure 7 Rapid uptake and degradation of ^{125}I-labelled 39 kDa protein by rat MH_1C_1 cells at 37 °C

Pre-chilled monolayers of MH_1C_1 cells were placed at 37 °C, and a pre-warmed solution of 28 nM ^{125}I-labelled 39 kDa protein was added to initiate internalization. At each time point, dishes were cooled directly on ice, and the overlying buffer was removed and precipitated with TCA as described in the Materials and methods section: ●, TCA-soluble radioactivity (internalized and degraded ligand) in the extracellular media; △, amount of cell-associated radioactivity as determined after lysis of the cell monolayers; ○, the sum of both cell-associated and extracellular degraded ligand. Each point is the mean of triplicate determinations. Similar results were seen in three independent experiments.

protein. This complex of ^{125}I-labelled 39 kDa protein, t-PA and LRP was of very high apparent molecular mass, as before, remaining largely in the stacking gel during electrophoresis (Figure 5, arrows). When these same samples were electrophoresed in the presence of 2-mercaptoethanol to cleave the cross-linker, each resulted in a single radiolabelled band corresponding to the exogenously added ^{125}I-labelled 39 kDa protein (arrowhead).

In order to determine if cross-linking of the 39 kDa protein to the MH_1C_1 cell surface generates an epitope for recognition by the anti-t-PA antibody, similar experiments were performed with ^{125}I-labelled 39 kDa protein bound and cross-linked to the MH_1C_1 cell surface, but in the absence of exogenously added unlabelled t-PA (Figures 5e and 5f). In this case, the high-molecular-mass complex of ligand-associated radioactivity was immunoprecipitable only with anti-LRP and anti-(39 kDa protein) antibodies, and not with the anti-t-PA antibody. We therefore conclude that the simple cross-linking of radiolabelled 39 kDa protein to the MH_1C_1 cell surface does not change its specificity for either the anti-t-PA or anti-(39 kDa protein) antibodies. A number of additional co-binding and cross-linking experiments, performed using ^{125}I-t-PA and unlabelled 39 kDa protein, gave similar results (not shown). These results, taken together with those presented above, lead us to conclude that the 39 kDa protein and 5-PA can bind simultaneously to LRP on the MH_1C_1 cell surface.

Rapid uptake and degradation of ^{125}I-labelled 39 kDa by MH_1C_1 cells

Recent studies in our laboratory have shown that MH_1C_1 cells can rapidly endocytose and degrade t-PA [18]. To investigate the ability of MH_1C_1 cells similarly to endocytose the 39 kDa protein, single-cycle uptake and degradation experiments were performed. Figure 6 shows the results from a typical experiment. As expected, all ligand-associated radioactivity is initially located at the cell surface. Within 3 min of warm-up, however, 80% of this population becomes intracellular (protease-resistant) or is dissociated into the media as TCA-insoluble (undegraded ligand) radioactivity. The intracellular levels of ligand reach a peak value of 50% of total at approx. 10 min. Concurrently, the TCA-soluble radioactivity, representing internalized and subsequently degraded ligand, appears in the overlying medium initially at 10 min, with plateau levels reached at between 45 and 60 min. This kinetic pattern of uptake and degradation of a single cohort of pre-bound ^{125}I-labelled 39 kDa ligand is identical with that observed for ^{125}I-t-PA with these MH_1C_1 cells.

Rate of 39 kDa-protein uptake by MH_1C_1 cells

In order to determine the rate at which the 39 kDa protein is catabolized by MH_1C_1 cells, pre-cooled cell monolayers were placed at 37 °C, and ligand uptake was initiated by the addition of a pre-warmed solution of 28 nM ^{125}I-labelled 39 kDa protein. At various times over the course of 2 h, dishes of cells were cooled on ice, and the amount of both cell-associated and extracellular processed ligand was determined as described above. Figure 7 shows the results from a typical experiment. The amount of cell-associated ligand increases rapidly, reaching a plateau at approx. 40 min after the transition in temperature. The amount of extracellular degraded ligand exhibits a lag of approx. 20 min, after which there begins a linear increase which lasts the duration of the experiment. The sum of both cell-associated and extracellular degraded ligand also increases linearly, with a slope identical with that for extracellular degraded ligand alone. The slopes of each of these parameters is a measure of the rate of ligand uptake and degradation at 37 °C and was determined to be 52 fmol/min per 10^6 cells. We have therefore

demonstrated a pattern of rapid catabolism of the 39 kDa protein by MH_1C_1 cells.

DISCUSSION

Our laboratory has previously demonstrated the existence of a PAI-1-independent t-PA uptake and degradation pathway on rat MH_1C_1 hepatoma cells [18], mediated largely through an interaction with the LRP receptor [6,18]. A 39 kDa protein has been shown to inhibit binding of specific ligands to LRP, including t-PA [6], *Pseudomonas* exotoxin A [8], α_2-macroglobulin [16], and apo E-enriched β-VLDL [16]. We therefore sought to define more specifically the interaction between the 39 kDa protein and MH_1C_1 cells, and the effect of this interaction on t-PA binding and catabolism.

Our data demonstrate both specific and saturable binding of the ^{125}I-labelled 39 kDa protein to a single kinetic binding species on the rat MH_1C_1 cell surface. Cross-linking studies using ^{125}I-labelled 39 kDa protein have shown that at least part of this specific interaction is mediated by an association with the LRP receptor. The 39 kDa protein can strongly inhibit t-PA binding to MH_1C_1 cells with an apparent K_i value of 0.5 nM. Additional cross-linking experiments suggest a direct interaction between these three molecules, with individual 39 kDa and t-PA molecules able to specifically associate with the same LRP receptor. Nanomolar concentrations of ^{125}I-labelled 39 kDa protein are rapidly endocytosed at 37 °C from the overlying buffer, with internalized ligand subsequently processed and released from the cells as TCA-soluble radioactivity. Finally, continuous-uptake experiments suggest a rate of processing of ^{125}I-labelled 39 kDa ligand of 52 fmol/min per 10^6 cells at 37 °C, implying receptor recycling to the plasma membrane (see below).

Specific binding studies with the ^{125}I-labelled 39 kDa protein demonstrate the existence of a single kinetic species of high-affinity receptor on the MH_1C_1 cell surface (Figure 1a). Binding data from a number of independent experiments demonstrate similar binding affinities for the MH_1C_1 cell surface for both the 39 kDa protein and t-PA (Tables 1a and 1b). There is, however, a substantial difference in the number of binding sites for t-PA and for 39 kDa protein per cell. MH_1C_1 cells typically bind between 5 and 7 times more ^{125}I-labelled 39 kDa protein than ^{125}I-t-PA.

The rate of uptake of the ^{125}I-labelled 39 kDa protein by MH_1C_1 cells, as measured by continuous-uptake experiments at 37 °C, is 52 fmol/min per 10^6 cells (Figure 7). Given that there are approx. 380000 39 kDa-protein-binding sites per cell (Table 1a), and given that the linear rate of uptake is reached at approx. 20 min after the transition in temperature, we calculate that the total population of cell surface receptors would be occupied within approx. 12 min (Figure 7). In fact, linear uptake of the radiolabelled 39 kDa protein continues well beyond 12 min, until at least 120 min after the temperature transition. Although it is unlikely that protein synthesis *de novo* results in the replacement of occupied receptors on the plasma membrane [22], it is possible that a large intracellular pool of the 39 kDa-protein receptor exists, which is available for rapid recruitment to the cell surface. To investigate this possibility, simultaneous binding experiments were performed with untreated cells and cells preincubated in 0.1% saponin for 30 min. In the presence of the detergent saponin, the plasma membrane and intracellular vesicular membranes are permeabilized to molecules of at least 160 kDa [24]. Saponin preincubation should therefore allow binding of the ^{125}I-labelled 39 kDa protein to an otherwise unavailable intracellular receptor pool. Data for saturation-binding experiments performed in both the presence and the absence of saponin are summarized in Table 1(c). These data demonstrate the existence of an intracellular pool of receptor, approximately equal in size to the population present on the plasma membrane. This pattern of distribution is similar to that seen for a number of other receptors, including the asialoglycoprotein receptor [22,24], the transferrin receptor [20], the LDL receptor [25] and the epidermal growth factor receptor [26]. Thus, in order to support a constant rate of 39 kDa-protein uptake for 2 h after the transition in temperature (Figure 7), a total cell complement of receptor at least 5–6-fold larger than that actually detected in the presence of saponin would be required. We therefore conclude that the 39 kDa-protein receptor recycles intracellularly, while delivering multiple rounds of 39 kDa-protein ligand from the plasma membrane.

Since the 39 kDa protein has a strong affinity for heparin, we sought to determine if ^{125}I-labelled 39 kDa protein binding to MH_1C_1 cells occurs in part via an interaction with the proteoglycans of the extracellular matrix. Specific binding of ^{125}I-labelled 39 kDa protein to MH_1C_1 cells was not inhibited in the presence of an excess of free heparin sulphate, nor did pretreatment of cell monolayers with heparinase I prevent specific 39 kDa-protein binding (results not shown). Furthermore, although binding of the 39 kDa protein to heparin is Ca^{2+}-independent, 39 kDa-protein specific binding to the MH_1C_1 cell surface is entirely Ca^{2+}-dependent (results not shown). We therefore sought to investigate the MH_1C_1-cell-specific 39 kDa-protein-binding species. Since a high-affinity association between the 39 kDa protein and LRP had previously been demonstrated [1,2], we wished to determine if 39 kDa-protein binding to MH_1C_1 cells resulted from an interaction with this receptor. Cross-linking studies demonstrate a specific interaction between ^{125}I-labelled 39 kDa protein and LRP (Figure 2). Furthermore, the interaction of unlabelled 39 kDa protein with [^{35}S]methionine-labelled cells appeared to be mediated almost entirely through an association with the LRP receptor (Figure 3). We therefore conclude that 39 kDa-protein binding MH_1C_1 cells occurs predominantly through an interaction with the LRP receptor.

Analysis of the cDNA sequence of the LRP receptor reveals a series of 31 cysteine-rich complement-type repeats [27,28] similar to those implicated in the binding of LDL to the LDL receptor [29]. The presence of such a large number of these repeats suggests the possibility of numerous independent ligand-binding domains within a single LRP receptor molecule. This possibility is further supported by evidence showing that LRP interacts specifically and simultaneously with a number of diverse ligands. Bu et al. [6] were able to demonstrate simultaneous binding of individual t-PA and α_2-macroglobulin molecules to the same LRP receptor. We have demonstrated a similar finding by simultaneously cross-linking both the 39 kDa protein and t-PA to the same LRP receptor on the MH_1C_1-cell surface (Figure 5). Our model of LRP function therefore assumes the presence of multiple independent ligand-binding domains.

Since the 39 kDa protein has been shown to inhibit binding of many of the previously described LRP ligands, it is likely that the 39 kDa protein shares an affinity for the binding sites of each of these molecules. One would therefore expect the 39 kDa protein to bind MH_1C_1 cells with a several-fold molar excess over each of the other LRP-specific ligands. Our binding data are in agreement with this hypothesis, with 5–7 times more ^{125}I-labelled 39 kDa protein binding to MH_1C_1 cells than, for instance, ^{125}I-t-PA binding (see Tables 1a and 1b). A model describing a series of independent ligand-binding domains, each of which can accommodate a single 39 kDa protein molecule, would require that the 39 kDa protein inhibits binding of each of the LRP-

specific ligands, whereas none of these ligands inhibit more than a fraction of 39 kDa-protein specific binding. This presumption is supported by data showing that the 39 kDa protein readily inhibits binding of α_2-macroglobulin [16], apo E-enriched β-VLDL [16], *Pseudomonas* exotoxin A [8], and t-PA (Figure 4), whereas no known LRP ligand has yet been shown to inhibit more than a fraction of specific 39 kDa-protein binding to the MH_1C_1 cell surface [6].

An implicit assumption of this model is that all MH_1C_1-cell-specific 39 kDa-protein-binding sites are binding sites on the LRP receptor. Although cross-linked complexes of unlabelled 39 kDa protein and radiolabelled MH_1C_1 cellular proteins appear to contain exclusively the larger subunit of LRP (Figure 3), we cannot rule out the possibility that, among a number of 39 kDa specific binding proteins, LRP is preferentially cross-linked to this ligand. In order to demonstrate that LRP is the major 39 kDa-protein binding species, one must first show that affinity-purified anti-(rat LRP) antibodies can inhibit all specific 39 kDa-protein binding to MH_1C_1 cells. Unfortunately, we at present do not have high-quality affinity-purified antibodies to the rat LRP receptor. Furthermore, our model would imply that specific 39 kDa-protein binding would be inhibited to a greater extent by several LRP-specific ligands acting in concert, rather than by any single ligand acting in isolation. Evaluation of these assumptions should allow for the generation of a clearer model of LRP and 39 kDa-protein interaction, with implications for our understanding of the catabolism of other LRP-specific ligands at the physiological level.

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