The Syndecan Family of Proteoglycans

Novel Receptors Mediating Internalization of Atherogenic Lipoproteins In Vitro

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Abstract

Cell-surface heparan sulfate proteoglycans have been shown to participate in lipoprotein catabolism, but the roles of specific proteoglycan classes have not been examined previously. Here, we studied the involvement of the syndecan proteoglycan family. First, transfection of CHO cells with expression vectors for several syndecan core proteins produced parallel increases in the cell association and degradation of lipoproteins enriched in lipoprotein lipase, a heparan-binding protein. Second, a chimeric construct, FcR–Synd1, that consists of the ectodomain of the IgG Fc receptor Ia linked to the highly conserved transmembrane and cytoplasmic domains of syndecan-1 directly mediated efficient internalization, in a process triggered by ligand clustering. Third, internalization of lipase-enriched lipoproteins via syndecan-1 and of clustered IgGs via the chimera showed identical kinetics ($t_{1/2}$ = 1 h) and identical dose-response sensitivities to cytochalasin B, which disrupts microfilaments, and to genistein, which inhibits tyrosine kinases. In contrast, internalization of the receptor-associated protein, which proceeds via coated pits, showed a $t_{1/2}$ < 15 min, limited sensitivity to cytochalasin B, and complete insensitivity to genistein. Thus, syndecan proteoglycans can directly mediate ligand catabolism through a pathway with characteristics distinct from coated pits, and might act as receptors for atherogenic lipoproteins and other ligands in vivo. (*J. Clin. Invest.* 1997. 100:1611–1622.) Key words: atherosclerosis • lipoprotein lipase • heparan sulfate proteoglycans • endocytosis • receptor

Introduction

Physiologically important pathways in both hepatic and arterial catabolism of atherogenic lipoproteins remain incompletely characterized. For example, the clearance of remnant lipoproteins by the liver is mediated in part through a LDL receptor–independent pathway (3–5) that has been referred to as the remnant receptor (3), though it has eluded definitive characterization (6). Accumulation of intracellular and extracellular lipid within the arterial wall is undiminished in the absence of LDL receptors (see reference 7), but can be only partly explained by known receptors. LDL receptor–independent pathways mediate about one-third of LDL removal from plasma in normal humans and all removal in patients homozygous for receptor-negative familial hypercholesterolemia (8, 9). Nevertheless, the search for additional receptors, particularly in the liver, has had a long and difficult history.

Currently, there are two molecules under general consideration as potential receptors for atherogenic lipoproteins. The first is proteoglycans: heparan sulfate proteoglycans (HSPGs)[1] in the liver (10, 11) and chondroitin sulfate proteoglycans and HSPGs in the arterial wall (7, 12, 13). It has long been known that several lipid-binding proteins, such as lipoprotein lipase (LpL) (14), apo E (15, 16), and hepatic lipase (17, 18), avidly adhere to heparin and to HSPGs. Early studies indicated that LpL can bridge between lipid-rich chylomicrons and endothelial HSPGs, thereby mediating cell-surface attachment of the lipoproteins (19). Subsequently, it was suggested that LpL might be the ligand responsible for hepatic clearance of chylomicrons, though no information was presented about the nature of the putative cellular recognition site (20). In 1986, Oswald et al. demonstrated that HSPGs can participate in cellular uptake of triglyceride-phospholipid emulsions enriched in apo E, though they dismissed a physiologic role for this phenomenon (16, 21).

We previously reported that LpL could enhance LDL receptor–independent cellular binding, as well as lysosomal degradation, of LDL and lipoprotein(a) by bridging between the atherogenic lipoproteins and the heparan sulfate (HS) side chains of cell-surface HSPGs (10, 22). It is a structural action

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Portions of this work were presented at the 66th and 68th Scientific Sessions of the American Heart Association (1, 2).

Some of this work was done when I.V. Fuki, K.M. Kuhn, I.R. Lomazov, and K.J. Williams were in the Department of Biochemistry, Medical College of Pennsylvania, Philadelphia, PA 19129.

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; LpL, lipoprotein lipase; LRP, LDL receptor–related protein; mLDL, methylated low density lipoprotein; RAP, receptor-associated protein.

of LpL: enzymatically inactive protein still retains this property (10). The presence of LpL within the space of Disse (23) and within the arterial wall (24, 25), two sites of LDL receptor–independent catabolism of lipoproteins, support the physiologic importance of this pathway. Subsequent work confirmed the existence of the LpL-proteoglycan pathway and extended it to VLDL and to protein-free emulsions (26–28). Also, the physiologic significance of the ability of apo E to promote HSPG-mediated catabolism of lipoproteins was strongly emphasized by Ji et al. (11), which has been supported in other studies, particularly of type III hyperlipoproteinemia (29, 30). Promotion of remnant catabolism in vitro by hepatic lipase has been shown to require cell-surface HSPGs, presumably owing to bridging (31). Hepatic lipase–facilitated catabolism of remnant lipoproteins has been demonstrated in vivo (32, 33), although the precise role of HSPGs in this process remains to be determined.

The other molecule under serious consideration as an additional lipoprotein receptor is the LDL receptor–related protein (LRP). Because it resembles several LDL receptors cobbled together, it became an attractive candidate for the chylomicron remnant receptor (for review see references 34 and 35). LRP has been reported to be the high molecular mass band observed in cross-linking experiments and on ligand blots of cellular homogenates probed with either apo E– (36, 37) or LpL-enriched (38) particles. Studies in vitro and in vivo supporting a role for LRP in lipoprotein catabolism include competition studies using the receptor-associated protein (RAP) (36, 39–41), which blocks the binding of all ligands to LRP (36); competition studies with activated α_2-macroglobulin (39, 42, 43); inhibitory studies using anti-LRP antibodies (36, 39, 44, 45); and genetic manipulation of LRP expression (46, 47).

Some studies, however, may leave room for additional mechanisms. For example, RAP is a known ligand for heparin (48), and it may (49) or may not (50) bind HSPGs. Thus, competition by RAP may not always distinguish between LRP- versus direct HSPG-mediated catabolism. Also, some work has failed to find substantial inhibition of lipoprotein catabolism by RAP (51–55). The ability of activated α_2-macroglobulin to block remnant catabolism in vivo is controversial (40, 42, 43, 53, 56), and competition in vitro is generally poor (16, 39, 42). Finally, genetic elimination of LRP expression in one experimental system reduced the catabolism of LpL by only one-third, and the remaining two-thirds was attributed to HSPGs (47).

There is now general agreement that removal of cell-surface HSPGs substantially impairs the uptake of LpL-, apo E–, or hepatic lipase–enriched lipoproteins in vitro (6, 10, 31, 34), and similar results have been obtained after heparinase treatment in vivo (53, 57, 58). Complex hypotheses involving lipoprotein transfer from HSPGs to LRP have been proposed to accommodate a role for both molecules (6, 34, 39). Several studies, however, are consistent with the existence of direct HSPG-mediated catabolism of lipoproteins (51, 52) and other ligands (47, 59–62), independent of LRP.

Based upon our initial observation of at least two metabolically distinct HSPG-mediated pathways, one mediating the degradation of ligand within 4 h and the other not (10), we now sought to identify the roles of specific classes of HSPGs in LDL receptor–independent binding, internalization, and degradation of lipoproteins. Here, we examined the syndecan family, a major class of cell-surface HSPGs (63–65) present on the perisinusoidal membrane of hepatocytes (66, 67) and expressed by vascular cells (68, 69) and macrophages (70, 71). This proteoglycan family is characterized by a single transmembrane domain and cytoplasmic tail, both of which are highly conserved amongst family members and across animal species (63–65, 72). As a model ligand, we used LpL-enriched ^{125}I-labeled–methylated LDL (LpL/^{125}I-mLDL), which binds HSPGs but does not interact with LDL receptors. Our results demonstrate that the syndecans function as receptors that directly mediate binding, internalization, and efficient lysosomal delivery of ligands through a pathway that can be triggered by clustering of the transmembrane and cytoplasmic domains.

Methods

Preparation of reagents. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Molecular biological enzymes were purchased from New England Biolabs Inc. (Beverly, MA). Oligonucleotides were synthesized by BioServe Biotechnologies, Ltd. (Laurel, MD).

Bovine LpL (EC 3.1.1.34) was purified from cow's milk by heparin-agarose chromatography (73) with minor modifications. All preparations were assayed for protein mass and enzymatic activity (74). Thrombospondin (TSP) was purified from human platelets (75). Whole human IgG and goat F(ab′)$_2$ against the Fab region of human IgG were purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Purified recombinant RAP was kindly provided by Dr. Dudley Strickland (American Red Cross, Rockville, MD) (76). Radioiodinated IgG and RAP were prepared using Iodobeads (Pierce Chemical Company, Rockford, IL). LDL was isolated from fresh human plasma by ultracentrifugation ($1.019 < d < 1.063$ g/ml), then radioiodinated by the iodine monochloride method (77). The ^{125}I-LDL was reductively methylated (^{125}I-mLDL) to modify $\sim$ 30% of the lysine residues, thereby abolishing LDL receptor binding (78).

Preparation of expression vectors for the core proteins of syndecan family members. All subcloning procedures were performed by standard methods (79).

Human syndecan–1 (also known as a human syndecan): the expression vector, pMAMneo-hsyn (63, 80), was digested with NheI and XhoI to excise the complete coding sequence for the core protein of human syndecan–1. The digest was fractionated on a 1% Agarose gel and the $\sim$ 1,300-bp syndecan–1 fragment was excised and purified using the Qiaex gel extraction kit from QIAGEN Inc. (Chatsworth, CA). The NheI and XhoI 5′ overhangs were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase–I. The product of this reaction was then ligated into the mammalian expression vector pEUK-C1 (Clontech, Palo Alto, CA), which had been previously linearized with SmaI and treated with calf intestinal alkaline phosphatase. After transformation of competent S.E. DH5α *E. coli*, one clone containing a sense-oriented syndecan-1 cDNA insert was identified by restriction and Agarose gel analysis. This construct, called pEUK–hSynd1, contained in the following order (5′ $\rightarrow$ 3′): the SV40 late promoter, two viral introns, the syndecan-1 cDNA in the multiple cloning site, then an SV40 polyadenylation signal.

Rat syndecan–2 (also known as rat fibroglycan): the C17-Bluescript construct (81) was used as a template to amplify the complete coding sequence for the core protein of rat syndecan–2 by PCR using the primers 5′-CgTCTAgAATgCgggTACgAgCCACgT-3′ (sense) and 5′-CgTCTAgAgACACTAAgTgggAgT-3′ (anti-sense). These primers create a stronger context for the ATG codon (82), eliminate the short poly(A^+) sequence, and introduce XbaI restriction sites. The resultant 668-bp PCR product was purified, digested with XbaI, and ligated into the pEUK-C1 expression vector. One plasmid containing a sense-oriented cDNA construct, called pEUK-rSynd-2, was identified by restriction and Agarose gel analysis.

Rat syndecan–4 (also known as rat ryudocan or rat amphiglycan):

the vector pNWS144 (68) was digested with BamHI and HindIII to excise the complete coding sequence for the core protein of rat syndecan–4. The ~ 630-bp band was purified and the 5′ overhangs filled in, as described above. The syndecan-4 fragment was then blunt-end ligated into the pEUK-C1 expression vector, which had been linearized previously with SmaI. A plasmid containing a sense-oriented cDNA construct, called pEUK–rSynd4, was identified by restriction and gel analysis.

Preparation of an expression vector for a chimera consisting of the ectodomain of the IgG-Fc receptor–Ia linked to the cytoplasmic and transmembrane domains of the syndecan-1 core protein. The vector pCD64 p135 (83), which contains the full-length human cDNA for the IgG-Fc receptor Ia (FcR), also known as CD64, was used as a template to amplify the coding region for essentially the entire ectodomain by PCR using the primers 5′-CgTCTAgATTTCACTgCTCCCACCA-3′ (sense) and 5′-AggAgTTggTAACTggAggCCA-3′ (anti-sense). The sense primer corresponded to a sequence beginning 30 bp upstream of the FcR start codon and introduced a new XbaI restriction site. The anti-sense primer consisted solely of sequence complementary to the cDNA, ending with the codon for Pro^{288}.

Using pMAMneo-hsyn (63, 80) as template, the coding region for the transmembrane and cytoplasmic domains of the human syndecan–1 core protein was amplified by PCR using the primers 5′-gAggTgCTgggAggggTCATT-3′ (sense) and 5′-CTCggATCCgCgTCAggCATAgAATT-3′ (anti-sense). The sense primer consisted solely of sequence from the cDNA, beginning with the codon for Glu^{251}. This hydrophilic extracellular residue, which is immediately adjacent to the transmembrane domain, was included to ensure that this domain would assume the proper conformation. This single extracellular residue is insufficient for any known function of the ectodomain (64, 65, 84). The anti-sense primer corresponded to a sequence from 11 bp upstream to 3 bp downstream of the termination codon and introduced a new BamHI restriction site.

For both PCR reactions, Vent polymerase was used, thereby producing blunt ends at the Pro^{288} and Glu^{251} codons. The PCR products were digested with XbaI and BamHI, respectively, and simultaneously ligated into the pEUK-C1 vector digested previously with the same two restriction enzymes. This construct, called pFcR–Synd1, contained the 5′ portion of the coding region for the human FcRI ending at Pro^{288} linked to the 3′ portion of the coding region for the human syndecan–1 core protein beginning at Glu^{251}. The construct was verified by restriction and Agarose gel analysis and by sequencing the entire insert.

Transfection of Chinese hamster ovary (CHO) cells. The wild-type CHO cell line, CHO-K1, obtained from the American Type Culture Collection, Rockville, MD (ATCC #CCL 61), was maintained in Ham's F-12 medium supplemented with 10% FBS. Transfections of CHO cells were performed using the calcium phosphate precipitation method (79) with minor modifications. Each construct of interest was cotransfected at a ratio of 24:1 with pMAM-neo, a plasmid containing a neomycin resistance gene. Stably transfected cells were then selected by incubation for about 3 wk in Ham's F-12 with 10% FBS, supplemented with 280 μg of active G418/ml. To control for possible random variations amongst transfected cell lines, the mixed (nonclonal) population and three positive colonies were selected for each plasmid and expanded into cell lines.

Cellular uptake and degradation of ^{125}I-mLDL. Cell lines were grown in 15-mm wells to ~ 90% confluence in serum-supplemented media. For the experimental incubations, cells were changed to a serum-free 1:1 mixture of Waymouth's MD-705/1 and MEM supplemented with 0.2% fatty acid–free BSA, ^{125}I-mLDL (5 μg/ml), and either LpL (5 μg/ml) or a matching volume of lipase buffer (74). Cells were incubated for up to 5 h in these media at 37°C, then cell association and cell-specific degradation of ^{125}I-lipoproteins were assayed as described previously (74, 77). In some experiments, cell association was separated into cell-surface and intracellular fractions by incubating the cells for 30 min at 4°C in the presence of 10 mg heparin per milliliter, which releases surface-bound material (77).

To examine the time course of ligand internalization and degradation, LpL and ^{125}I-mLDL were incubated with cells in serum-free medium for 1 h at 4°C, to allow surface binding without further catabolism. The cells were washed to remove unbound material. Fresh media at 37°C with no ligands were then added, and incubations at 37°C were continued for the indicated times. Assays for surface-bound, intracellular, and degraded ligand were then performed. In addition, TCA-precipitable radioactivity in the media was quantified, as an indication of retroendocytosis or desorption from the cell surface during the incubation at 37°C.

To examine intracellular processes involved in ligand catabolism, specific inhibitors were added simultaneously with medium at 37°C to cells with surface-bound LpL/^{125}I-mLDL complexes. These included cytochalasin B (0–400 μM), which disrupts the cytoskeleton (85); freshly prepared genistein (0–400 μM), a tyrosine kinase inhibitor (86); its inactive 7-glucoside analogue, genistin (87); and chloroquine (100 μM), an inhibitor of lysosomal proteases (77).

All results for ^{125}I-lipoprotein catabolism were normalized to cellular protein (88), which averaged 65 μg per well for both wild-type CHO-K1 cells and transfected CHO lines. LpL-dependent catabolism was calculated by subtracting the values obtained in the absence of LpL (LpL-independent catabolism) from those obtained in the presence of LpL (total catabolism). We obtained essentially identical results when LpL-independent values were determined by assessing catabolism in the presence of a low concentration of heparin (66 μg/ml) that is known to displace LpL from cell-surface HSPGs (10).

Cellular uptake and degradation of ^{125}I-IgG, and the effects of ligand clustering. To verify expression of a functional FcR ectodomain in CHO cells transfected with pFcR-Synd1, transfected and control cells were incubated at 37°C for 5 h with 5 μg of ^{125}I-IgG per milliliter, and then total cellular protein and cell-associated ^{125}I-radioactivity were determined.

To examine effects of ligand clustering, transfected cells were incubated with ^{125}I-IgG at 4°C for 1 h, to allow cell-surface binding. Cells were rinsed at 4°C to remove unbound ligand, then prewarmed media at 37°C were added, without or with our clustering agent (unlabeled goat $F[ab']_2$ against human IgG Fab; 4 μg/ml final concentration). This agent was chosen to avoid interference with ^{125}I-IgG binding to the FcR ectodomain. Cells were then incubated at 37°C for up to 5 h, then chilled to 4°C and rinsed. To separate surface-bound from intracellular material, cells were incubated at 4°C for 2 min in acidified PBS (pH, 2.5), a standard method to release IgG from cell-surface Fc receptors (89). We obtained comparable results when surface-bound material was released by incubation at 4°C for 30 min with *Streptomyces griseus* protease (10 mg/ml). Degraded ligand was assayed by the release of ^{125}I-tyrosine into the media (77). Studies with cytochalasin B, genistein, and chloroquine were performed according to the protocols described above.

Cellular uptake and degradation of ^{125}I-RAP. Cells were incubated for 15 min at 37°C in media supplemented with ^{125}I-RAP (3 μg/ml), with or without an excess of unlabeled RAP (50 μg/ml). Surface-bound material was released by incubation of cells for 1 h at 4°C in PBS (pH 7.4) containing trypsin (50 μg/ml), proteinase K (50 μg/ml), and sodium EDTA (5 mM) (90). Material resistant to this treatment was considered to be intracellular, as described previously (90). All values presented for surface binding and internalization of ^{125}I-RAP were calculated by the difference between values in the absence and presence of excess unlabeled RAP (nonsaturable binding and internalization were about 20% of the total).

Statistical analyses. Each data point in the time–course and dose–response curves is the mean of duplicate determinations. All other results are given as mean±SEM, $n = 3$. Absent error bars when $n = 3$ indicate SEM values smaller than the drawn symbols. Each figure displays data from a representative experiment out of at least three independent studies. For comparisons between a single experimental group and a control, the unpaired, two-tailed t test was used. For comparisons involving several groups simultaneously, ANOVA was initially used. When the ANOVA indicated differences amongst the

groups, pairwise comparisons of each experimental group versus the control group were performed using the Dunnett q' statistic (91).

Results

Involvement of syndecan family members in ligand catabolism. We sought to examine the role of the syndecan family in cellular catabolism of lipoproteins. For these experiments, we used CHO cells, which are readily transfected and exhibit the lipase-proteoglycan pathway for lipoprotein catabolism (10, 22). The mixed lines stably transfected with our expression constructs, pEUK–hSynd1, pEUK–rSynd2, and pEUK–rSynd4, were designated as CHO–Synd1, CHO–Synd-2, and CHO–Synd4, respectively. Northern blots under high stringency to avoid interference from endogenous hamster messages verified expression of the appropriate mRNA in each line, though quantitation indicated several-fold higher values in CHO–Synd1 than in CHO–Synd2 or CHO–Synd4 (data not shown).

We next compared the ability of LpL to enhance ^{125}I-mLDL catabolism by transfected and wild-type cells. During a 5-h incubation, LpL-dependent cell association and degradation of ^{125}I-mLDL by CHO–Synd1 cells were double the values in control CHO-neo cells (Fig. 1). LpL-dependent cell association and degradation in the CHO–Synd1 cells were > 95% inhibited by 66 μg heparin per milliliter, a concentration that selectively blocks binding to heparan sulfate side chains (10). Furthermore, competition studies indicated that TSP, a known ligand for syndecan-1 (92, 93), almost completely abolished LpL-dependent cell association and degradation of ^{125}I-mLDL by CHO–Synd1 cells (Fig. 2).

CHO–Synd2 and CHO–Synd4 cell lines also demonstrated enhanced LpL-dependent cell association (147.8±8.1% and 127.9±11.9% of control, respectively) and degradation (138.9± 8.8% and 164.5±18.6% of control) of ^{125}I-mLDL, but to a lesser extent than the CHO–Synd1 cells, perhaps in part because of unequal expression of the transfected constructs or because of intrinsic differences amongst family members (64, 65). Enhanced LpL-dependent catabolism of ^{125}I-mLDL was also found in clonal lines subcultured after transfection with pEUK–hSynd1, pEUK–rSynd2, and pEUK–rSynd4 (data not shown).

These results indicate that expression of syndecan family members substantially enhanced cellular binding of LpL-lipoprotein complexes, which was followed by increased internalization and degradation. Based on previous literature, there are two possible scenarios for internalization of ligands bound to these cell-surface HSPGs: either internalization is directly mediated by the syndecan family itself, or internalization requires cooperation with an auxiliary receptor, such as LRP, that is actually responsible for bringing the material into the cells. In all transfected lines, however, the increases over control cells in LpL-dependent cell association were accompanied by similar percentage increases in LpL-dependent degradation, suggesting that no auxiliary molecules were rate-limiting in the internalization and degradation of the LpL-lipoprotein complexes.

Direct internalization of ligands triggered by clustering of the syndecan transmembrane and cytoplasmic domains. The competition study in Fig. 2 indicates that TSP and LpL-lipoprotein complexes share cell-surface binding sites and a catabolic pathway. This is of particular interest, because the mechanism of TSP internalization is known to involve clustering

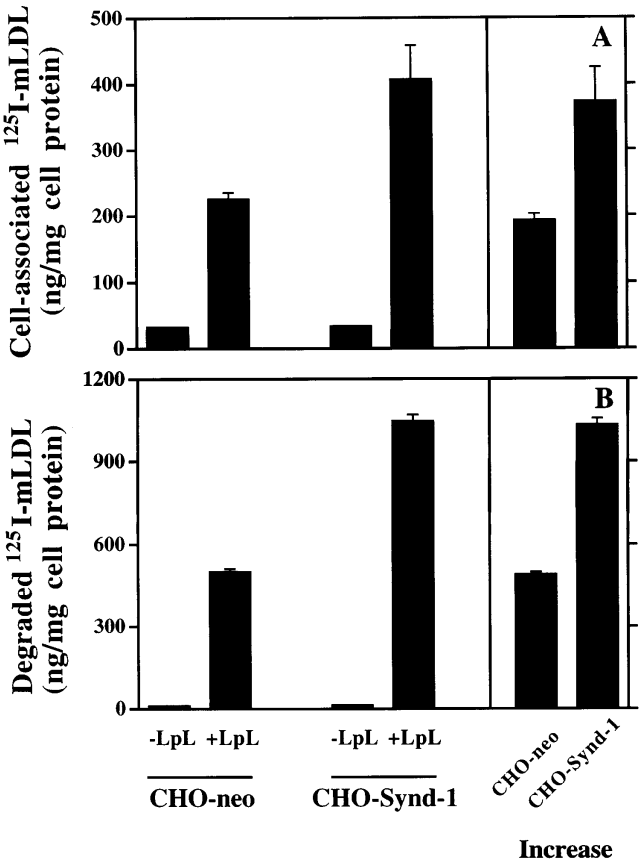

Figure 1. Participation of syndecan-1 in LpL-dependent catabolism of ^{125}I-mLDL. CHO-K1 cells were stably transfected with pMAM-neo alone (*CHO-neo*) or with an expression construct for the syndecan-1 core protein (*CHO-Synd1*). Cells were incubated for 5 h at 37°C in the presence of ^{125}I-mLDL, without (−*LpL*) or with (+*LpL*) 5-μg LpL per milliliter. Cell association (*A*) and cell-specific degradation (*B*) of ligand were measured, and the results are displayed by the four lefthand columns in each panel. The two righthand columns in each panel (*Increase*) show the absolute increases in cell association or degradation attributable to LpL. This experiment was performed simultaneously with studies of cell lines transfected with syndecan-2 and -4 expression constructs (see text for data), and ANOVA indicated differences in LpL-dependent cell association ($P < 0.01$) and degradation ($P < 0.005$) amongst the four cell lines tested. In the data displayed, LpL-dependent cell association and degradation were each significantly higher in the CHO–Synd1 cells than in the CHO-neo controls ($P < 0.01$ and $P < 0.001$, respectively, by Dunnett's test).

of its cell-surface HSPG binding sites (94). Notice that TSP (95) and LpL-lipoprotein complexes are both large, multimeric ligands, which would be expected to provoke receptor clustering.

Distinguishing between direct internalization via syndecan itself versus the transfer of ligand to LRP has been impeded by the lack of specific reagents: most ligands that bind HS side chains and become internalized have also been reported to interact with LDL receptor family members on ligand blots (e.g., LpL, apo E, RAP, TSP, and hepatic lipase). Therefore, we generated a chimeric construct, in which the proteoglycan ectodomain of syndecan-1 is replaced by the ectodomain of a receptor with the following characteristics: (*a*) a similar archi-

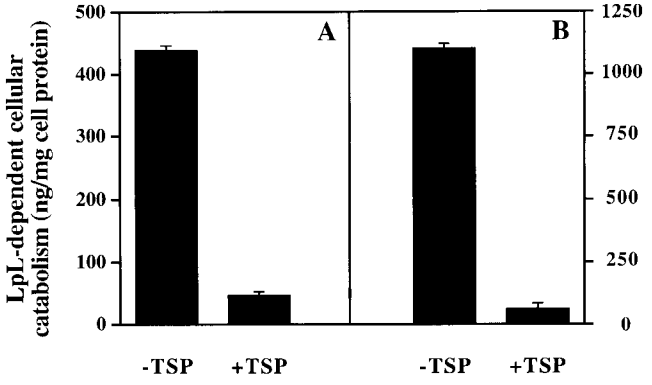

Figure 2. Competitive inhibition by thrombospondin of LpL-dependent catabolism of ^{125}I-mLDL. LpL-dependent cell association (*A*) and degradation (*B*) of ^{125}I-mLDL by CHO–Synd1 cells were assessed during a 5-h incubation at 37°C in the absence (−*TSP*) or presence (+*TSP*) of 100-μg thrombospondin per milliliter. Each displayed value was calculated as the difference between ^{125}I-mLDL catabolism with and without LpL. Thrombospondin had no effect on values in the absence of LpL and no effect on LpL adsorption to the lipoprotein (data not shown). Thrombospondin significantly decreased LpL-dependent cell association and degradation of the lipoprotein ($P < 0.001$).

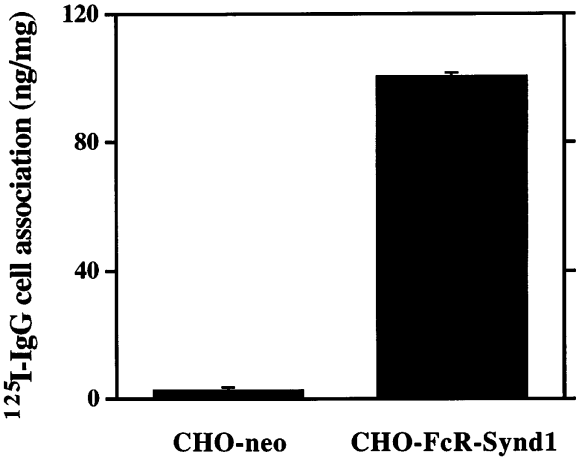

Figure 3. Association of ^{125}I-IgG to control CHO cells and to CHO cells transfected with the pFcR-Synd1 chimeric expression construct. CHO-K1 cells transfected with pMAM-neo alone (*CHO-neo*) or with the FcR-Synd1 chimera (*CHO-FcR-Synd1*) were incubated for 5 h at 37°C with human ^{125}I-IgG. Displayed is total cell-associated radioactivity.

tecture to the syndecan core protein family, namely, a single NH_2-terminal ectodomain anchored by a single transmembrane domain; (*b*) specificity for readily available ligands that do not interact with proteoglycans, LDL receptor family members, or any other molecules on the surface of wild-type CHO cells; (*c*) an ability to bind both monomers and multimers; (*d*) existence of reagents that can cluster ligands and receptors; and (*e*) no spontaneous polymerization of the ectodomain. The human IgG Fc receptor Ia (FcR) fulfilled all of these requirements (83, 96), and its ectodomain has been used successfully to make chimeras to study other intracellular domains (97). Our chimeric expression construct, pFcR–Synd1, contained the coding region for the FcR ectodomain linked to the syndecan-1 transmembrane and cytoplasmic domains, thereby allowing us to investigate the role of these syndecan domains in directing cellular catabolism of bound ligand, without confounding effects from ligand transfer.

Fig. 3 displays total cell association of ^{125}I-IgG to CHO-neo and CHO-FcR–Synd1 cells after a 5-h incubation at 37°C. Essentially no material bound to CHO-neo cells (Fig. 3) or to untransfected wild-type CHO-K1 cells (data not shown), indicating that there are no endogenous receptors on the surface of these cells with which this ligand can interact. In contrast, substantial amounts of ^{125}I-IgG became associated with the CHO-FcR–Synd1 cells, indicating functional expression of the FcR ectodomain in our chimera.

Next, we sought to determine if ligand bound to our chimera becomes internalized. ^{125}I-Labeled IgG was bound to the surface of CHO-FcR–Synd1 cells at 4°C, unbound material was washed away, and then the cells were incubated for 45 min in unlabeled media, either at 4°C or at 37°C, in the absence or presence of our clustering agent (goat F[ab′]$_2$ against human IgG Fab). For cells kept at 4°C, only 7.7±0.4% of cell-associated ligand was resistant to an acid wash, consistent with the absence of internalization (Fig. 4, 4°C). Incubation of cells at 37°C in the absence of the clustering agent failed to substantially increase the amount of internalized ligand (Fig. 4, 37°C, without clustering). In contrast, incubation of cells for 45 min at 37°C in the presence of our clustering agent resulted in the intracellular delivery of ∼ 50% of the surface-bound ligand (Fig. 4, 37°C, with clustering). Thus, the chimeric receptor itself is able to mediate internalization directly, in the absence of any other cell-surface molecules that could bind its ligand, and the signal for efficient internalization is receptor clustering. These results indicate that the transmembrane and cytoplas-

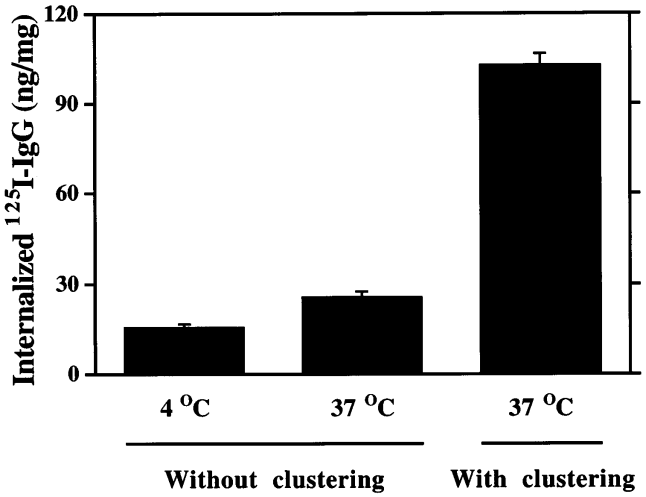

Figure 4. Internalization of surface-bound ^{125}I-IgG by CHO-FcR–Synd1 cells, without and with ligand clustering. CHO-FcR–Synd1 cells with surface-bound ^{125}I-IgG were incubated for 45 min at 4°C or 37°C, as indicated, in the absence (*Without clustering*) or presence (*With clustering*) of goat F[ab′]$_2$ against human IgG Fab. A brief acid wash at 4°C was then used to separate intracellular material (displayed) from material remaining on the cell surface. Addition of the goat F[ab′]$_2$ at 4°C had no effect on these parameters (data not shown).

mic domains of syndecan-1 are sufficient to direct ligand internalization.

Direct internalization versus ligand transfer. Although the above results indicate that direct internalization by syndecan-1 can occur, they do not exclude a substantial contribution from ligand transfer to an auxiliary receptor. Therefore, we compared the pathway mediated by HSPGs, in which ligand transfer has been proposed to play a central role, versus the pathway mediated by our chimera, in which ligand transfer is impossible. A key measurement is the kinetics: a substantial role for ligand transfer should be reflected in a significantly faster internalization of ligands bound to HSPGs than to the chimera.

We found that the catabolism of surface-bound LpL/^{125}I-mLDL complexes by CHO–Synd1 cells was relatively rapid: intracellular accumulation showed a broad peak at about 1 h, and degradation was mostly completed by 2.5 h (Fig. 5 *A*). Catabolism of surface-bound ^{125}I-IgG by CHO-FcR–Synd1 cells in the presence of the clustering agent proceeded at a similar rate (Fig. 5 *B*). Total internalization of ligand (intracellular accumulation plus degradation) by CHO–Synd1 and CHO-FcR–Synd1 cells showed utterly indistinguishable kinetics, with $t_{1/2}$s in both cases of 1 h (Fig. 5 *C*). For both ligands, there was no significant increase in TCA-precipitable radioactivity in the media after the initial 15 min at 37°C, indicating the absence of retroendocytosis (data not shown). Slight differences in the kinetics of degradation after internalization (compare Fig. 5, *A* and *B*) are likely to be a consequence of differences in the affinities of these ligand for their receptors, as described in other systems (98). For comparison with the kinetics of coated pit-mediated internalization, which is very rapid (99, 100), we found that > 85% of cell-associated ^{125}I-labeled native LDL and ^{125}I-RAP were internalized by CHO–Synd1 cells after 15 min at 37°C, consistent with previous reports (99–101) and considerably different from the time courses in Fig. 5. Therefore, these results indicate that efficient internalization mediated by syndecan-1 may be entirely explained by clustering of its transmembrane and cytoplasmic domains, with no detectable role for ligand transfer, and that this pathway might be distinct from classical coated pit endocytosis.

To test specifically for the involvement of LRP, we examined the ability of unlabeled RAP to interfere with the catabolism of LpL-enriched lipoproteins. The controversy over RAP binding to HSPGs (49, 50) allows several explanations when there is competitive inhibition by RAP: blockage of ligand transfer to LRP, blockage of ligand binding to HSPGs, or blockage of ligand clustering with minimal effects on HSPG binding (see reference 94 and Figs. 4 and 5). Nevertheless, because RAP is an unambiguous ligand for LRP (36), such experiments are straightforward to interpret when RAP does not competitively block ligand catabolism. In our system, saturating concentrations of RAP (50 μg/ml) did not affect the surface binding of LpL/^{125}I-mLDL complexes to CHO–Synd1 cells at 4°C (value was 98.1±1.8% of the no-RAP control). A separate experiment to examine the catabolism of LpL/^{125}I-mLDL complexes by CHO–Synd1 cells during a 5-h incubation at 37°C indicated that unlabeled RAP had no effect on surface binding (value was 94.8±1.5% of control), intracellular accumulation (106.8±1.6% of control), or degradation (100.7±1.2% of control). Thus, consistent with our conclusion from Fig. 5, there was no detectable role for LRP in lipoprotein catabolism in our experimental system.

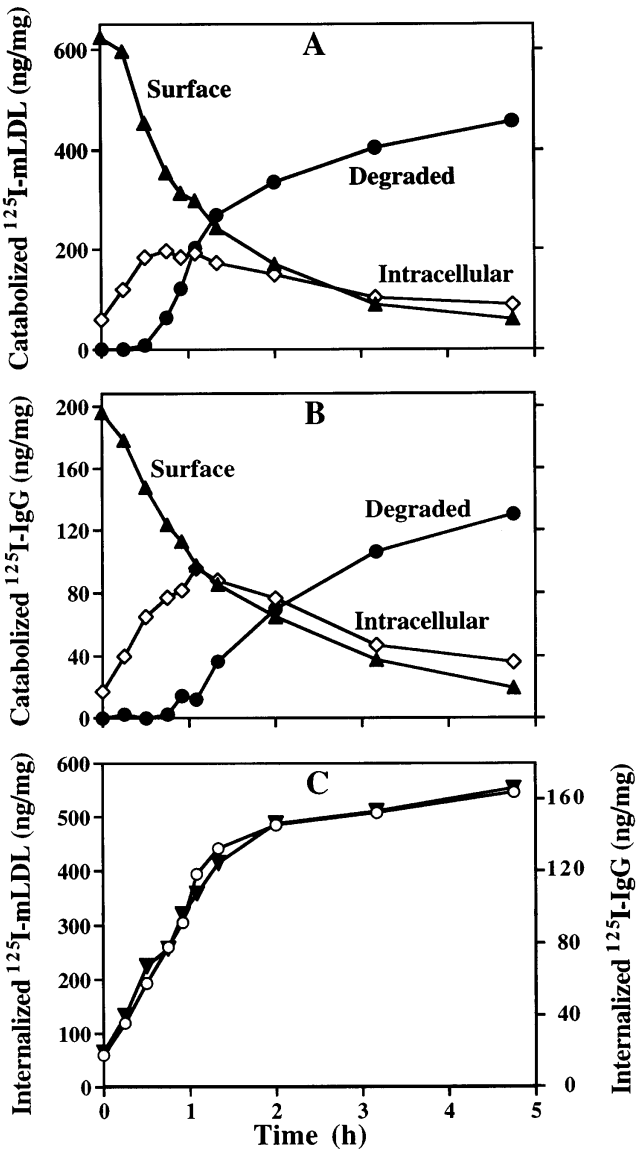

Figure 5. Time course of the catabolism of surface-bound ligands by CHO-Synd1 and CHO-FcR–Synd1 cells. (*A*) LpL-dependent catabolism of surface-bound ^{125}I-mLDL by CHO–Synd1 cells. Values displayed are the differences between catabolism in the presence and absence of LpL. Virtually identical kinetics were seen with CHO-neo cells, although all values were ~ 50% of those displayed here (data not shown). (*B*) Catabolism of surface-bound ^{125}I-IgG by CHO-FcR–Synd1 cells, in the presence of the clustering agent. Without clustering, ^{125}I-IgG was catabolized by these cells at roughly one-fifth the rate. In *A* and *B*, measurements of ^{125}I-ligand remaining on the cell surface (*filled triangles*), intracellular accumulation of ligand (*open diamonds*), and degraded ligand (*filled circles*) are displayed. The amount of ^{125}I-labeled material lost into the medium during the first 15 min was subtracted from the surface-bound quantitations displayed for t = 0. (*C*) Comparison of total internalization (intracellular accumulation plus degradation) of surface-bound ligand by CHO–Synd1 cells (*open circles* and y-axis scale on left; data from *A*) and CHO-FcR–Synd1 cells (*inverted filled triangles* and y-axis scale on right; data from *B*). Notice that the number of ligand particles internalized by the two cell lines at each time point was nearly identical (e.g., the last time point in *C* indicates 1.1 pmol of internalized ^{125}I-mLDL and 1.0 pmol of internalized ^{125}I-IgG per milligram cell protein).

We next sought to determine the cellular destination of the direct syndecan pathway. Chloroquine (150 μM) inhibited > 85% of the degradation of LpL/^{125}I-mLDL complexes by CHO–Synd1 cells and of clustered ^{125}I-IgG by CHO-FcR–Synd1 cells, indicating the involvement of lysosomes.

Cellular mechanisms involved in the direct catabolism of ligands via syndecan versus the LRP pathway. Clustering of syndecan-1 has been reported to cause specific interactions between its cytoplasmic tail and actin microfilaments (64, 85, 102). Therefore, we examined the effects of cytochalasin B, which disrupts the actin cytoskeleton (85). We found substantial inhibition of ligand internalization via syndecan-1 and the FcR–Synd1 chimera during a 45-min incubation at 37°C, and the dose-response curves were superimposable (Fig. 6 *A*, *open circles* and *inverted filled triangles*). Ligand degradation via syndecan-1 and the FcR–Synd1 chimera during a 5-h incubation at 37°C were > 90% inhibited by cytochalasin B (Fig. 6 *B*), supporting a role for the cytoskeleton in the entire catabolic pathway.

Parallel experiments on the LRP pathway were complicated by two issues. First, the kinetics of ^{125}I-RAP internalization are markedly different from internalization via the syndecan pathway (see above). Therefore, we examined ^{125}I-RAP internalization during 15-min incubations, rather than during the 45-min incubations used for syndecan-1 and the chimera. Second, cytochalasin B, which was added when cells were brought to 37°C (see Methods), may not act instantaneously. Therefore, to control for possible delays in the onset of effects of cytochalasin B on LRP, we incubated CHO–Synd1 cells with ^{125}I-RAP during the first 15 min after cells were brought to 37°C (t = 0–15 min) and during the last 15 min (t = 30–45 min) of the same 45-min period when we examined the syndecan pathway. The dose–response curves for the effects of cytochalasin B on ^{125}I-RAP internalization during these two 15-min periods (Fig. 6 *A*, ✕ and ✚) do not match the curves for syndecan-1 or the FcR–Synd1 chimera. At the lower concentrations of cytochalasin B, the pathways mediated by syndecan-1 and the chimera were at least twice as sensitive as the LRP pathway (Fig. 6 *A*).

Because the syndecan family transmembrane/cytoplasmic domain possesses four highly conserved tyrosyl residues (63–65) that may be phosphorylated (103), we examined the effects of genistein, a tyrosine kinase inhibitor with broad specificity (86, 104).[2] Addition of genistein to CHO-FcR–Synd1 cells substantially inhibited the internalization of ^{125}I-IgG during a 45-min incubation at 37°C in the presence of the clustering agent (Figure 7 *A*, *inverted filled triangles*), consistent with a requirement for tyrosine kinase activity (see reference 89). In striking contrast, genestein had no effect at all on ^{125}I-RAP internalization during either of the 15-min periods that we examined (Fig. 7 *A*, ✕ and ✚). To examine effects on ligand degradation, we used both transfected cell types. To minimize fading of the genistein effect (104), we assessed degradation of surface-bound ligands after incubating the cells at 37°C for 2 h, an early time point at which degradation can be readily detected (Fig. 5). Genistein substantially inhibited ligand degradation via syndecan-1 and the chimera, and the dose–response curves were nearly identical (Fig. 7 *B*).

Thus, the pathways mediated by syndecan-1 and by the FcR–Synd1 chimera are essentially identical in their kinetics, lysosomal delivery of ligands, and dependence on cytoskeletal actin and tyrosine kinases, consistent with a central role for the

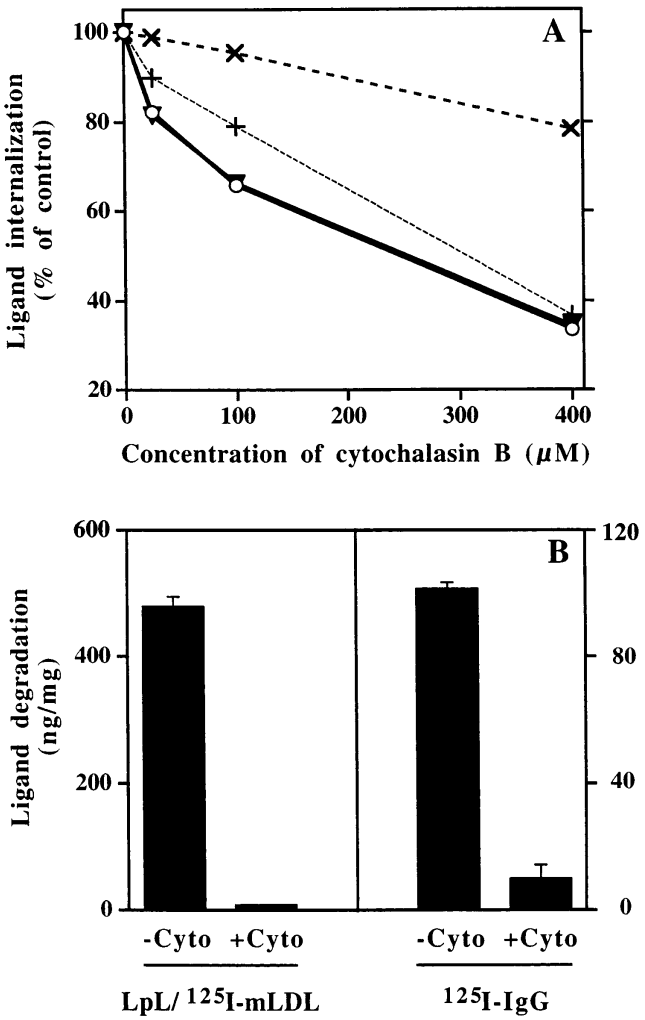

Figure 6. Effect of cytochalasin B on ligand catabolism by CHO–Synd1 and CHO-FcR–Synd1 cells. (*A*) Internalization of surface-bound LpL/^{125}I-mLDL by CHO–Synd1 cells (*open circles*) and clustered ^{125}I-IgG by CHO-FcR–Synd1 cells (*inverted filled triangles*) during a 45-min incubation at 37°C in the presence of the indicated concentrations of cytochalasin B. Values in the absence of cytochalasin B (100% on the y-axis) were 171.6±0.9 and 69.9±0.3 ng/mg for the two cell types, respectively. As a control for the general effects of this inhibitor on endocytosis, internalization of ^{125}I-RAP by CHO–Synd1 cells was examined from 0–15 min (✕) and from 30–45 min (✚) after the addition of the indicated concentrations of cytochalasin B. (*B*) Effect of 400-μM cytochalasin B on degradation of surface-bound ligands (*LpL/^{125}I-mLDL* and clustered *^{125}I-IgG*) by the two cell types. Cells with surface-bound ligands were incubated for 5 h at 37°C in medium supplemented with Me_2SO solvent alone (−*Cyto*) or with cytochalasin B (+*Cyto*).

2. Our initial studies indicated that both genistein and its inactive analogue, genistin, substantially enhanced the heparin-resistant association of LpL/^{125}I-mLDL complexes to CHO–Synd1 cells even at 4°C, a temperature that blocks all ligand internalization. Slightly over half of this material could be released by protease. There were, however, no effects of the inactive analogue on the acid-releasable or -resistant association of ^{125}I-IgG to CHO-FcR–Synd1 cells, nor on the cellular degradation of either LpL/^{125}I-mLDL or ^{125}I-IgG.

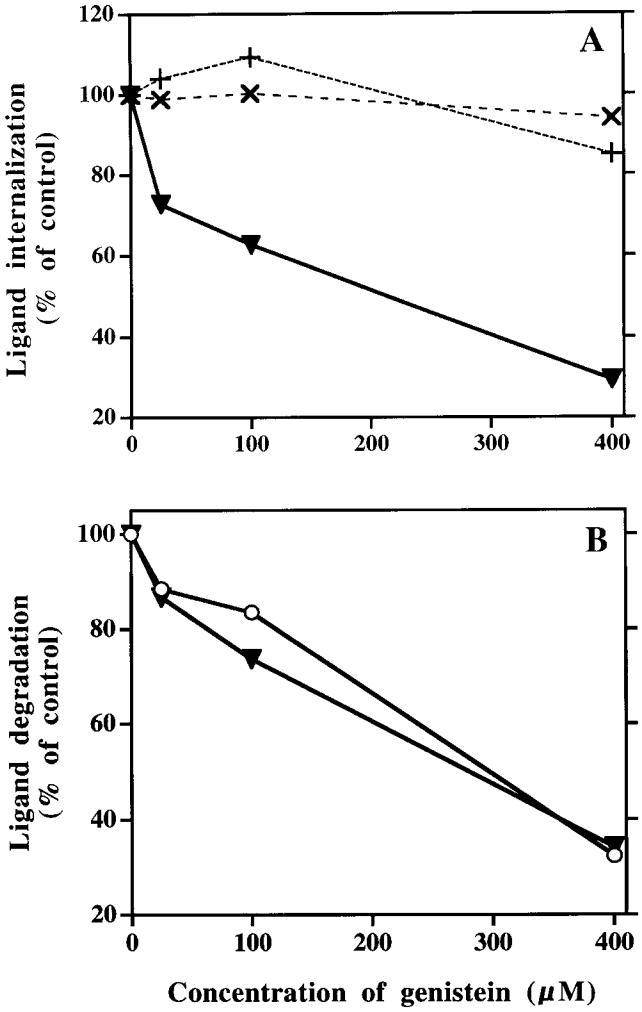

Figure 7. Effect of genistein on ligand catabolism by CHO–Synd1 and CHO-FcR–Synd1 cells. (*A*) Internalization of surface-bound, clustered ^{125}I-IgG by CHO-FcR–Synd1 cells (*inverted filled triangles*) during a 45-min incubation at 37°C in the presence of the indicated concentrations of genistein. The value in the absence of inhibitors (100% on the y-axis) is given in the legend to Fig. 6 *A* (experiments were done concurrently). As a control for the general effects of genistein on endocytosis, internalization of ^{125}I-RAP was examined from 0–15 min (✖) and from 30–45 min (✚) after the addition of the indicated concentrations of genistein. (*B*) Degradation of surface-bound ligand by CHO–Synd1 cells (*open circles*) and CHO-FcR–Synd1 cells (*inverted filled triangles*) during a 2-h incubation at 37°C in the presence of the indicated concentrations of genistein. Values in the absence of genistein (100% on the y-axis) were 386.8±4.7 and 65.7±7.3 ng/mg for the two cell types, respectively.

transmembrane and cytoplasmic domains in directing the catabolism of bound ligands. Moreover, key features of the syndecan-mediated pathway are distinct from the internalization of a coated-pit ligand, ^{125}I-RAP.

Discussion

Previous studies (10, 22, 26, 27) have established that cell-surface HSPGs are essential for LpL-dependent catabolism of atherogenic lipoproteins. Relying entirely on experiments with live cells, we have now identified specific proteoglycans belonging to the syndecan family that are participants in this pathway. Our results indicate that syndecan family members not only bind ligands at the cell surface, but are also capable of directly mediating efficient internalization and delivery to lysosomes, in a process that can be signaled by clustering of the transmembrane and cytoplasmic domains of the core protein. These results suggest that the syndecan family may be able to act as receptors for atherogenic lipoproteins and other ligands in vivo.

The syndecan family, like other cell-surface receptors, possesses a distinctive domain organization reflecting its function (64). All HS side-chains are attached to the core protein of syndecan-1 near the distal tip of its ectodomain (105). Thus, this ligand-binding region, which is unusual only in that it is carbohydrate and not protein, is located far from the cell surface, thereby facilitating the approach of large, bulky ligands, such as lipoproteins or thrombospondin multimers. Furthermore, the internal structure of HS chains places sulfate-rich, heparin-like domains away from the attachment site to the core protein (106), which could also favor the binding of large ligands. The region of the core protein that separates the HS attachment sites from the cell surface is poorly conserved (64, 65), consistent with a role as a spacer.

The transmembrane and cytoplasmic domains, which are highly conserved, mediate signaling and can direct the lysosomal catabolism of bound ligand. Once clustered, these domains appear to require cytoskeletal interactions (Fig. 6 and references 85 and 102) and tyrosine kinase activity (Fig. 7). The sequence around the second tyrosyl residue (Tyr^{286} of human syndecan-1) resembles consensus motifs for phosphorylation (64), consistent with a report that the syndecan-1 cytoplasmic tail can be tyrosine phosphorylated (103). The mechanism for syndecan clustering may involve the binding of large, multimeric ligands that would bridge between receptors, or it may involve spontaneous polymerization mediated by the side chains (107) or by the transmembrane domain in cooperation with a conserved tetrapeptide sequence immediately outside the cell (84). The major functional domains of syndecan are ancient structures that have been extraordinarily well-conserved through evolution, indicating their biologic importance: HS has been found in sea anemones (108), and the amino acyl sequences of the transmembrane and cytoplasmic domains are $>$ 50% identical between each vertebrate syndecan and the insect form (72).

Our kinetic studies (Fig. 5) suggest that the syndecan family may be responsible for the faster component of HSPG-mediated catabolism of lipoproteins in vitro (10). Preliminary studies indicate that perlecan, another cell-surface HSPG, may contribute to the slower component of HSPG-mediated lipoprotein catabolism (109). Perlecan interacts with the cell surface via specific core protein domains (110) that are unrelated to syndecans and therefore likely to account for the differences we observed in the catabolism of bound ligand. Because both syndecan-1 and perlecan are abundant within the hepatic space of Disse (64, 66, 67), we speculate that their combined participation might explain observed rates of LDL receptor–independent internalization of chylomicron remnants in vivo (53, 58, 111).

Several authors have advocated a major role for LRP in the cellular internalization and lysosomal delivery of LpL-, apo E–, or hepatic lipase–enriched atherogenic lipoproteins, spe-

cifically by the transfer of ligand from HSPGs to LRP as a prelude to internalization (6, 11, 34, 39). It seems unlikely, however, that cell-surface LRP, which cannot readily capture LpL-, apo E–, or hepatic lipase–enriched ligands in the absence of cellular HSPGs (11, 27, 31, 39, 49), would then be able to tear these ligands away from the HSGPs that did capture them. By contrast, the LDL receptor, which readily captures ligands independently of HSPGs (e.g., references 10 and 27), may be able to obtain ligands from HSPGs, and this process could account for the more rapid internalization of chylomicron remnants by normal livers than by livers lacking LDL receptors (53, 111).

For cell-surface LRP to have a role, however, there should be additional complexity, such as HSPG-induced changes in conformation leading to greater affinity of these ligands for cellular LRP, or simultaneous binding of ligands to HSPGs and LRP. The fact that LRP on the surface of cells does not readily bind or internalize LpL-enriched lipoproteins in the absence of HSPGs (27, 39), but purified LRP does (38, 39), suggests that cellular LRP might be capable of conformational changes leading to high-affinity binding of these ligands. The K_d for LpL binding to purified LRP is 18 nM (i.e., 1.8 μg/ml) (90), which, if achieved by LRP on the cell surface, would produce substantial internalization and degradation of ligand, even in the absence of nearby HSPGs. Transfer of another ligand, basic fibroblast growth factor, from HSPGs to the bFGF receptor has been reported (112, 113), but in that case, heparin-induced conformational changes have been demonstrated unambiguously (112). Nevertheless, it should be noted that several experimental approaches failed to find any involvement of LRP in our experimental system (see Figs. 5–7 and Results).

The time course of ligand internalization mediated by syndecan or the FcR–Synd1 chimera exhibited a $t_{1/2}$ of 1 h (Fig. 5 *C*), which is similar to previous reports of the internalization of LpL-enriched lipoproteins (51) and TSP (114), though not in all studies (26). These kinetics are not consistent with the direct involvement of coated pits, which are known to internalize surface-bound ligands with $t_{1/2}$s of 5–15 min (99–101). One explanation for this discrepancy would be ligand transfer with a $t_{1/2}$ of about 1 h, followed by near-instantaneous internalization via coated pits. This model, however, could not account for the identical kinetics exhibited by our chimera, in which ligand transfer is impossible. Another explanation would be clustering of syndecan or FcR–Synd1 with $t_{1/2}$ of about 1 h, followed by internalization via coated pits. Nevertheless, antibody-mediated clustering, which is essentially what we used with our FcR–Synd1 chimera, is not rate limiting for endocytosis via coated pits (115). Finally, syndecan-mediated internalization may not involve coated pits. In addition to the distinct kinetics of this pathway (Fig. 5), its sensitivity to cytochalasin B and to genistein are different from the coated-pit pathway for ^{125}I-RAP internalization (Figs. 6 *A* and 7 *A*). The properties of syndecan-mediated internalization that we observed are reminiscent of internalization via caveolae, which can proceed with a $t_{1/2}$ of 1 h and strongly depends on the cytoskeleton (116).

Based on our data and the previous literature, there are three possibilities for the catabolism of ligands that can bind to HSPGs: (*a*) binding, internalization, and lysosomal delivery mediated solely by HSPGs, with no involvement of other cell-surface molecules, such as LDL receptor family members (see Figs. 4–7 and references 52, 59, 61, 62); (*b*) parallel pathways, in which HSPGs and non-HSPGs each independently bind and internalize ligands (47); and (*c*) a cooperative pathway, in which HSPGs must present their ligands to auxiliary cell-surface molecules that then mediate internalization (6, 34), although, as noted above, this process may require conformational changes in the ligands or receptors. It is likely that the relative roles of these three mechanisms will depend on the ligands, cell types, HS side chain structures, and specific HSPG core proteins involved. A central question in lipoprotein physiology will be the contributions of these three mechanisms in the liver and within the arterial wall in vivo. If the time course displayed in Fig. 5 and the inhibitor studies in Figs. 6 and 7 indicate a pathway truly distinct from coated pits, then it would be informative to determine the proportions of lipoproteins or other ligands that are internalized in vivo via coated versus uncoated membrane structures.

Overall, our data indicate that the syndecan family of HSPGs directly mediates binding, internalization, and lysosomal delivery of its ligands. Thus, syndecans act as receptors in vitro and might represent a physiologically important component of LDL receptor-independent catabolism of ligands in vivo (117).

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