Transmembrane and cytoplasmic domains of syndecan mediate a multi-step endocytic pathway involving detergent-insoluble membrane rafts

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Syndecan heparan sulphate proteoglycans directly mediate a novel endocytic pathway. Using Chinese hamster ovary cells expressing the human syndecan 1 core protein or a chimaeric receptor, FcR-Synd, consisting of the ectodomain of the IgG Fc receptor Ia linked to the transmembrane and cytoplasmic domains of syndecan 1, we previously reported that efficient internalization is triggered by ligand clustering, requires intact actin microfilaments and tyrosine kinases, proceeds with a $t_{1/2}$ of approx. 1 h and is distinct from coated-pit pathways. We have now examined the involvement of cholesterol-rich, detergent-insoluble membrane rafts. On clustering, ^{125}I-labelled IgG bound to FcR-Synd rapidly became insoluble in cold Triton X-100, well before endocytosis. Insolubility of clustered FcR-Synd ligand did not require the syndecan ectodomain, linkage of the cytoplasmic tail to the cytoskeleton, or energy-dependent cellular metabolism. Pretreatment of cells with cyclodextrin to deplete cholesterol from rafts abolished insolubility of the clustered ligand and inhibited endocytosis in a dose-responsive fashion. Similar results were obtained with ^{125}I-labelled lipoprotein lipase bound to authentic cell-surface syndecan. In contrast, the 39 kDa receptor-associated protein (RAP), a coated-pit ligand, was more than 80 % soluble in cold Triton even after internalization; cellular cholesterol depletion failed to substantially affect the internalization of ^{125}I-RAP. Overall, our results indicate a multi-step endocytic process consisting of ligand binding, clustering, energy-independent lateral movement into detergent-insoluble membrane rafts and finally recruitment of actin and tyrosine kinases to bring the ligands into the cell.

Key words: caveolae, cholesterol, coated pits, endocytosis, lipoprotein.

INTRODUCTION

Cell-surface heparan sulphate proteoglycans (HSPGs) participate in the catabolism of a wide variety of ligands, including lipoproteins, enzymes, infectious agents, platelet secretory products and proteins implicated in Alzheimer's disease (reviewed in [1]). We previously reported that the syndecan family of HSPGs directly mediates the internalization and the lysosomal delivery of ligands through a novel endocytic pathway that is independent of coated pits [2]. By several criteria, endocytosis via syndecan was identical with endocytosis via a chimaeric receptor, FcR-Synd, consisting of the ectodomain of the IgG Fc receptor Ia linked to the highly conserved transmembrane and cytoplasmic domains of human syndecan 1, thereby indicating a dependence on these domains [2]. Efficient endocytosis by this pathway is triggered by ligand clustering, requires intact actin microfilaments and tyrosine kinases and proceeds with a $t_{1/2}$ of approx. 1 h. Because coated pits are not involved [2], in the present study we sought to determine whether this pathway depends on other known regions of the plasma membrane.

Two lines of evidence led to the suspicion that cholesterol-rich, detergent-insoluble membrane rafts might be involved [1]. First, on clustering, syndecan has been shown to become insoluble in cold Triton X-100. However, this insolubility has been variably attributed to linkage of the syndecan cytoplasmic tail to the cytoskeleton [3,4], linkage of the heparan sulphate side chains on the syndecan ectodomain to insoluble cell-surface molecules or extracellular matrix [5], or a possible role for the syndecan transmembrane domain on the basis of the persistence of detergent insolubility after deletion of all except the first four intracellular amino acyl residues [6]. Secondly, fibroblast growth factor 2, that had been modified to bind only HSPGs but not its kinase-linked receptor, was reported to undergo internalization via flask-shaped structures and caveolae [7], although the specific classes of cell-surface HSPGs mediating this internalization were not determined. Such anatomic structures are known to be rich in cholesterol and sphingomyelin and insoluble in cold Triton (reviewed in [8–12]).

In the present study, we again used the FcR-Synd chimaera expressed in Chinese hamster ovary (CHO) cells as an experimental tool because this system employs a ligand, ^{125}I-IgG, that is unaffected by detergent, does not donate or remove cholesterol from the membrane, permits the easy control of clustering and exhibits no interactions with other molecules on the outer surface of these cells [2]. Whenever possible, we performed parallel experiments with ^{125}I-labelled lipoprotein lipase (^{125}I-LpL), a lipid-free dimeric ligand that binds cell-surface HSPGs [13] and undergoes endocytosis [14,15]. To contrast with coated-pit-mediated endocytosis, we also studied ^{125}I-labelled 39 kDa receptor-associated protein (^{125}I-RAP), a lipid-free ligand for the LDL receptor-related protein [16–18].

Portions of this work were presented at the XIth International Symposium on Atherosclerosis, Paris, France [19], and at the

Abbreviations used: CHO, Chinese hamster ovary; FcR-Synd, a chimaera of the ectodomain of the IgG Fc receptor Ia linked to the transmembrane and cytoplasmic domains of human syndecan 1; HSPGs, heparan sulphate proteoglycans; LpL, lipoprotein lipase; RAP, 39 kDa receptor-associated protein.

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71st Scientific Sessions of the American Heart Association, Dallas, TX, U.S.A. [20].

MATERIALS AND METHODS

Preparation of reagents

Reagents were obtained as described [2], with the following additions. Triton X-100 and 2-hydroxypropyl-β-cyclodextrin were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.). RAP, which is the human counterpart of mouse heparin-binding protein 44 [21] and a universal inhibitor for ligand binding to the LDL-receptor-related protein [22], was expressed as a recombinant protein in bacteria, with the use of a construct provided by Dr D. Strickland [23]. Bovine milk LpL (EC 3.1.1.34) was purified by heparin–agarose chromatography as described in [24,25] with minor modifications, then radioiodinated and repurified by heparin-affinity chromatography [26].

Cellular catabolism of ^{125}I-labelled ligands

CHO cell lines transfected with an expression vector for the FcR-Synd chimaera (CHO-FcRSynd) were described previously [2]. These cells were grown to confluence in Ham's F-12 medium supplemented with 10 % (v/v) fetal bovine serum and rinsed, then incubated at 4 °C for 1 h with 5 μg/ml ^{125}I-labelled non-immune human IgG in serum-free Ham's F-12 medium with 0.2 % BSA, to allow cell-surface binding. Cells were rinsed at 4 °C to remove unbound ligand, then fresh media at 4 or 37 °C were added, without or with our clustering agent [unlabelled goat $F(ab')_2$ against human IgG Fab; 4 μg/ml final concentration]. Cells were then incubated at 4 or 37 °C for up to 1 h, then placed at 4 °C for analysis.

Two additional ligands were also studied. One was ^{125}I-LpL, which is a ligand for authentic syndecan HSPGs. This ligand was surface-bound at 4 °C to CHO cells expressing the human syndecan-1 core protein (CHO-Synd1) [2], followed by an incubation at 4 or 37 °C, as above. Because LpL forms dimers and higher oligomers [27], no clustering agent was needed. The other ligand was ^{125}I-labelled RAP, which is catabolized by CHO cells through LDL-receptor-related protein [28], a coated-pit receptor. ^{125}I-RAP was present continuously during incubations at 4 or 37 °C, as described previously [2]. Because of the rapidity of coated-pit-mediated endocytosis [29,30], incubations with ^{125}I-RAP were limited to 15 min.

One group of wells was used for the assessment of the surface binding, internalization and degradation of labelled ligand. To separate surface-bound ligands from intracellular ligands, cells were chilled to 4 °C and then incubated in acidified PBS to release surface-bound IgG [2,31], 10 mg/ml heparin to release surface-bound ^{125}I-LpL [32], or a cocktail of proteases with EDTA to release cell-surface ^{125}I-RAP [2,33]. Lysosomal degradation of ^{125}I-labelled ligands was assayed by the release of ^{125}I-tyrosine into the media [34,35]. To assess the efficiency of endocytosis, we calculated ligand internalization as the sum of intracellular accumulation plus degradation, as described previously [2,15]. Internalization calculated in this fashion takes into account ligand still within the cells and also ligand taken up by cells but then degraded into amino acids, which the cells release to the culture medium. Because cells were exposed to labelled ligands for at most 1 h at 37 °C, only a small portion of the internalized material was degraded. Results for the surface binding and internalization of ^{125}I-labelled ligands were normalized to cellular protein content [36], which averaged 90 μg per 16 mm well and 410 μg per 35 mm well.

In a separate group of wells, solubility in cold Triton X-100 was assayed by the incubation of cells for 5 min at 4 °C in a 1 % (v/v) solution of chilled Triton X-100 3,5,6,20]. Solubilized material was assayed directly by ^{125}I radioactivity released into the solution. The residual material, identified as cold-Triton-insoluble, was dissolved in 0.1 M NaOH and quantified by γ-counting. Results are expressed as the percentage of total cell-associated ligand that was insoluble in cold Triton. Insolubility in warm Triton was assessed similarly, except that the incubation of cells in 1 % (v/v) Triton was conducted at 37 °C.

Metabolic inhibitors and compositional modifications of cells

Cytochalasin D (2 μM), a cytoskeletal disruptor [37], was added to cells for 30 min of preincubation at 37 °C, before allowing surface binding of ^{125}I-labelled ligands at 4 °C; once this inhibitor had been added it was kept present in all media for the rest of the experiment. The combination of 15 mM deoxyglucose with 15 mM NaN_3, which depletes cellular ATP [38], was added to cells in a similar fashion. Pretreatment with β-cyclodextrin (2, 10 or 50 mM), which removes unesterified cholesterol from the cell membrane [39,40], involved a 1 h incubation at 37 °C, immediately followed by chilling the cells to 4 °C for the addition of ^{125}I-labelled ligands. Cells subsequently kept at 4 °C required no additional exposure to cyclodextrin, while cells warmed to 37 °C after the addition of labelled ligands received the compound in all subsequent incubation solutions to maintain membrane cholesterol depletion. To quantify the degree of cellular cholesterol loss, cellular lipids were extacted in propan-2-ol; unesterified and esterified cholesterol were then measured by the cholesterol oxidase method (Wako Diagnostics, Richmond, VA, U.S.A.) [41].

Statistical analyses

Each data point in the time-course curves is the mean for duplicate derminations. All other results are displayed as means $\pm$ S.E.M., $n = 3$. Error bars absent from Figures when $n = 3$ indicate S.E.M. values smaller than the drawn symbols. Standard errors for the differences between means of groups with equal n were calculated as the square root of the sum of the squares of the individual S.E.M. values. For comparisons between a single experimental group and a control, the unpaired, two-tailed t test was used. For comparisons involving several groups simultaneously, analysis of variance (ANOVA) was initially used. When the ANOVA indicated differences between the groups, one of two approaches was selected: either pairwise comparisons of each experimental group with a single control group were performed with the Dunnett q' statistic, or all possible pairwise comparisons between groups were performed with the Student–Newman–Keuls q statistic [42].

RESULTS AND DISCUSSION

We began by determining whether the FcR-Synd chimaera could become insoluble in cold Triton and, if insolubility did develop, whether there was a temporal relationship with ligand internalization. The ligand, ^{125}I-IgG, was bound to the surface of CHO-FcRSynd cells at 4 °C and unbound ligand was washed away; warm medium was then added, either without or with our clustering agent. Cells were incubated at 37 °C for 1 h, then chilled to 4 °C, after which insolubility in cold Triton and internalization were quantified. Figure 1(A) shows that substantial insolubility in cold Triton did develop, but only after clustering. As reported previously, clustering also stimulated internalization (Figure 1B). ^{125}I-LpL, the ligand for authentic

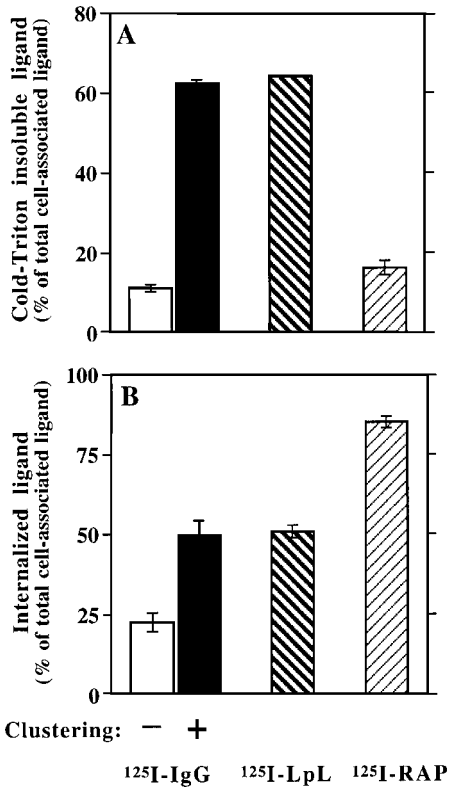

Figure 1 Insolubility in cold Triton (A) and internalization (B) of three cell-associated ligands

^{125}I-IgG was bound to the surface of CHO-FcR-Synd cells, then incubated for 1 h at 37 °C in the absence (−) or presence (+) of our clustering agent. ^{125}I-LpL was bound to the surface of CHO-Synd1 cells, then incubated for 1 h at 37 °C. ^{125}I-RAP was incubated with CHO-FcR-Synd cells for 15 min at 37 °C. Insolubility in cold Triton and internalization were then determined as described in the Materials and methods section. Total cell-associated ligand (100% on the *y*-axis) was 267.4 ± 13.1 ng/mg ^{125}I-IgG, 139.2 ± 4.6 ng/mg ^{125}I-LpL and 145.2 ± 0.4 ng/mg ^{125}I-RAP.

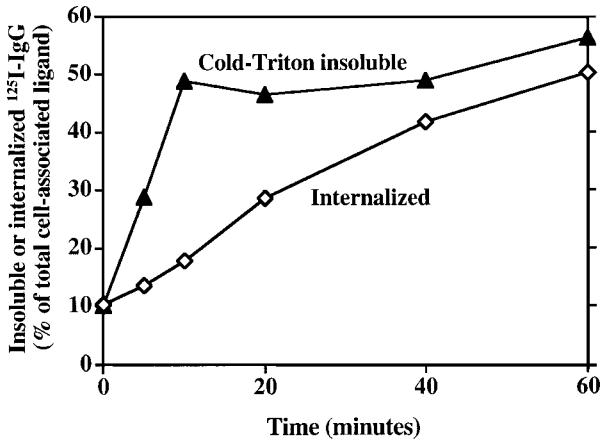

Figure 2 Time course for the development of insolubility in cold Triton and progression of endocytosis via clustered FcR-Synd

CHO-FcR-Synd cells with surface-bound ^{125}I-IgG were incubated for the indicated durations at 37 °C in the presence of our clustering agent; the insolubility of ^{125}I-IgG in cold Triton (▲) and internalization of ^{125}I-IgG (◇) were then assessed.

syndecan, showed essentially the same degree of insolubility in cold Triton (Figure 1A) and a similar rate of internalization (Figure 1B) to that of clustered ^{125}I-IgG bound to the chimaera (see [2]). In contrast, the internalization of ^{125}I-RAP, a coated-pit ligand, exhibited much more rapid internalization, as reported previously [2,43], but a trivial degree of insolubility in cold Triton, even after internalization (Figure 1). To examine the time courses for insolubility in cold Triton and endocytosis, we used the FcR-Synd chimaera, which permits the easy control of clustering. The effect of clustering was rapid: 50% of cell-associated ligand became Triton-insoluble in approx. 10 min, well in advance of endocytosis, which proceeded with a $t_{1/2}$ of approx. 60 min (Figure 2).

We next sought to examine the mechanism for the development of insolubility in cold Triton. Clearly, insolubility does not require the syndecan ectodomain, which our chimaera lacks. Thus the previous finding that the addition of heparin decreases syndecan insolubility [5] might reflect the disruption of syndecan clusters (see [44] on disruption of clustered HSPGs by heparin, and [1] for a discussion of mechanisms for syndecan clustering). To determine the role of linkage between the syndecan cytoplasmic tail and actin microfilaments in the development of insolubility in cold Triton [3,4], we used two methods. The first was the treatment of cells with cytochalasin D, which disrupts the cytoskeleton [37] and inhibits endocytosis via the FcR-Synd chimaera and syndecan [2]. However, this agent failed to decrease the cold-Triton insolubility of clustered ^{125}I-IgG/FcRSynd complexes or of ^{125}I-LpL bound to authentic syndecan, despite inhibiting endocytosis (Figure 3). The second method to examine the role of the cytoskeleton was the use of Triton X-100 at 37 °C, which can solubilize cholesterol-rich membrane rafts but not the actin cytoskeleton. At this temperature, only 24 ± 1.0% and 35 ± 1.2% respectively of surface-bound, clustered ^{125}I-IgG and syndecan-bound ^{125}I-LpL remained Triton-insoluble. Thus both methods indicate at most a minor role for the cytoskeleton in the development of insolubility in cold Triton. Depletion of intracellular ATP with deoxyglucose plus NaN_3 [38] did not inhibit the development of insolubility in cold Triton after ligand clustering (Figure 3), indicating an energy-independent process. Finally, we performed the clustering of surface-bound ^{125}I-IgG at 4 °C, incubated the cells for an additional 1 h at 4 °C, then assessed solubility in Triton X-100 at 4 °C, without ever warming the cells. Again, for both clustered ^{125}I-IgG bound to the FcR-Synd chimaera and ^{125}I-LpL bound to syndecan, 60–70% became insoluble in cold Triton (Figure 3). As expected, neither of these ligands was internalized at 4 °C (Figure 3). Taken together, these results indicate that insolubility in cold Triton after ligand clustering does not require the syndecan ectodomain, linkage of the syndecan cytoplasmic tail to the cytoskeleton, ligand internalization, or any active, energy-dependent cellular metabolism.

We next focused on the role of the plasma membrane. The insolubility of plasma membrane rafts in detergent depends on their unusual composition, which is rich in unesterified cholesterol. To determine whether the detergent insolubility of clustered ^{125}I-IgG/FcRSynd complexes or ^{125}I-LpL bound to syndecan is a membrane phenomenon, we depleted the membrane of cholesterol with 50 mM β-cyclodextrin [39,40]. Cholesterol depletion had no effect on the amount of either labelled ligand that initially bound to the cell surface (Table 1 legend), but largely abolished the development of insolubility in cold Triton (Table 1). Cholesterol depletion of the membrane also substantially interfered with ligand internalization (Table 1),

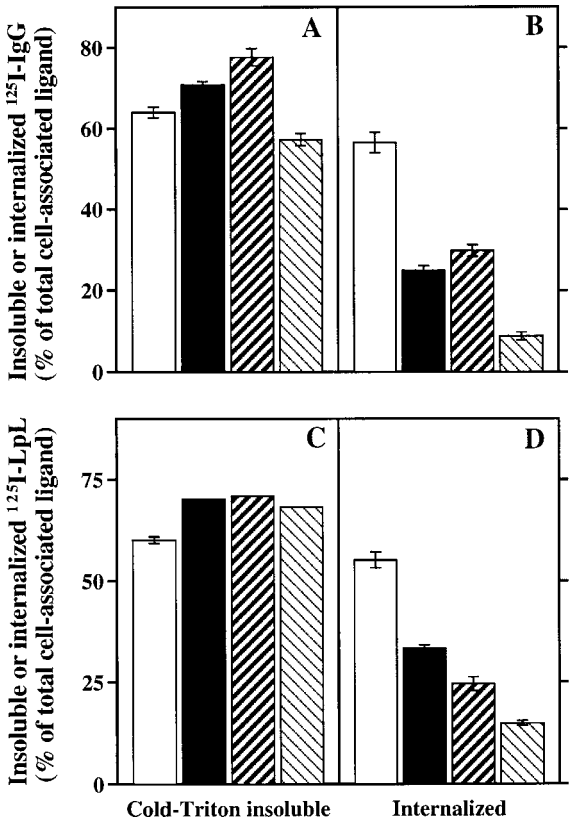

Figure 3 Role of the cytoskeleton and active cellular metabolism in the development of cold-Triton insolubility of ligands bound to FcR-Synd and to syndecan

Cold-Triton insolubility (left panels) and internalization (right panels) of clustered ^{125}I-IgG bound to the surface of CHO-FcR-Synd cells (**A**, **B**) and ^{125}I-LpL bound to the surface of CHO-Synd cells (**C**, **D**) were assessed as described in the legend to Figure 1. These procedures were performed in the absence of any metabolic inhibitors (control, white bars), in the presence of cytochalasin D (black bars) or azide with deoxyglucose (heavy-hatched bars), or by replacing the 1 h incubation at 37 °C with a 1 h incubation at 4 °C (light-hatched bars). None of these interventions affected the total amount of cell-associated ^{125}I-labelled ligands. In the absence of the clustering agent, the proportions of ^{125}I-IgG bound to control CHO-FcR-Synd cells were 9.9 ± 0.9% cold-Triton insoluble and 21.4 ± 1.0% internalized. In each panel, all values were significantly different from control ($P < 0.01$ by ANOVA then Dunnett's test), except for the black and lightly hatched bars in (**A**).

Table 1 Role of membrane cholesterol in the development of insolubility in cold Triton and the progression of endocytosis via different receptors

CHO cells expressing the appropriate receptor were pretreated without (−CD) or with (+CD) 50 mM β-cyclodextrin, to deplete cholesterol-rich membrane regions. The indicated ligands were bound to the cell surface and incubated at 37 °C, followed by assessments of insolubility in cold Triton and internalization. Values are given as percentages of total cell-associated ligand. Arithmetic differences between the absence and presence of β-cyclodextrin are also indicated (Δ); these were the only values to be subjected to statistical analysis. The incubations at 37 °C lasted for 1 h for clustered ^{125}I-IgG and ^{125}I-LpL, and 15 min for ^{125}I-RAP. Treatment of cells with cyclodextrins did not affect the initial binding of any of these ligands to their cell-surface receptors. *Significantly different ($P < 0.01$ by ANOVA then Student–Newman–Keuls test) from each of the other two Δ values. The Δ values from CHO-FcR-Synd compared with CHO-Synd cells were not statistically distinguishable.

Ligand		Insoluble in cold Triton (%)	Internalization (%)
Clustered ^{125}I-IgG on CHO-FcR-Synd cells	−CD	56.8 ± 1.8	51.7 ± 0.5
	+CD	14.2 ± 1.3	29.4 ± 0.6
	Δ	42.6 ± 2.2	22.3 ± 0.8
^{125}I-LpL on CHO-Synd1 cells	−CD	64.1 ± 0.2	55.2 ± 2.0
	+CD	22.7 ± 2.4	29.0 ± 1.6
	Δ	41.4 ± 2.4	26.2 ± 2.6
^{125}I-RAP on CHO-FcR-Synd cells	−CD	16.2 ± 0.1	71.7 ± 1.4
	+CD	6.6 ± 1.2	61.7 ± 1.1
	Δ	9.6 ± 1.2*	10.0 ± 1.8*

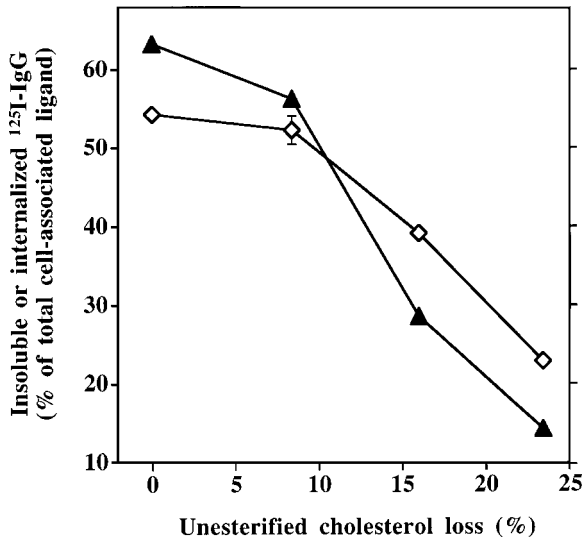

Figure 4 Effect of different degrees of cellular cholesterol depletion on the development of insolubility in cold Triton and the progression of endocytosis via clustered FcR-Synd

CHO-FcR-Synd cells were treated with 0 (control), 2, 10 or 50 mM β-cyclodextrin, as described in the Materials and methods section. The insolubility in Triton (▲) and internalization (◇) of surface-bound, clustered ^{125}I-IgG were assessed after a 1 h incubation at 37 °C. The *x*-axis displays the percentage depletion of cellular unesterified cholesterol relative to control cells, which contained 46.6 ± 3.3 μg of unesterified and 14.5 ± 0.7 μg of esterified cholesterol per mg of cell protein. In the absence of the clustering agent, the proportions of ^{125}I-IgG bound to control CHO-FcR-Synd cells were 8.3 ± 0.8% cold-Triton insoluble and 15.8 ± 3.3% internalized after 1 h at 37 °C. By linear regression, the displayed values for insolubility in cold Triton and internalization were strongly correlated ($r = 0.97$).

suggesting a causal connection between the initial development of detergent insolubility and subsequent endocytosis via this pathway. In contrast, treatment of cells with cyclodextrin had only minimal effects on the internalization of ^{125}I-RAP, whereas the insolubility of this ligand in cold Triton remained less than 20% with or without cyclodextrin (Table 1) (see [45,46]).

To determine the dose–response relationship of cellular cholesterol depletion to the inhibition of cold-Triton insolubility and endocytosis, we examined the effects of different concentrations of β-cyclodextrin. Figure 4 indicates that insolubility and endocytosis were inhibited together throughout the entire range of cellular unesterified cholesterol depletion. Insolubility and internalization showed a strong linear correlation (Figure 4 legend), supporting a causal relationship.

Finally, we sought to determine whether endocytosis could be accelerated by allowing maximal detergent insolubility to develop at 4 °C before warming the cells to 37 °C. On the basis of the kinetic data in Figure 2 (▲), ^{125}I-IgG that was clustered upon warming to 37 °C reached near-maximal detergent insolubility within 10 min. Thus pre-clustering at 4 °C to allow detergent insolubility to develop before warming should accelerate internalization by about 10 min. To permit the detection of this acceleration, we limited the incubation at 37 °C to 15 min, when the kinetic curve for endocytosis was steepest (Figure 2, ◇).

After 15 min at 37 °C, $24.2 \pm 0.9\%$ of surface-bound ^{125}I-IgG that had been clustered on warming became internalized, whereas $36.4 \pm 0.9\%$ of surface-bound ^{125}I-IgG that had been clustered beforehand at 4 °C became internalized ($P < 0.001$ by two-tailed t test). With the use of the curve in Figure 2 for internalization kinetics, these internalization values correspond to 14 and 30 min respectively, indicating a 16 min acceleration of endocytosis.

Overall, these results indicate a multi-step endocytic pathway involving cholesterol-rich membrane rafts. The process begins with ligand binding, then clustering of the syndecan transmembrane and cytoplasmic domains. Clustering provokes energy-independent lateral movement into cholesterol-rich, detergent-insoluble membrane rafts, an event followed by recruitment of the actin cytoskeleton and tyrosine kinases to bring the ligands into the cell.

Integral cell-surface receptors anchored by phosphatidylinositol have also been reported to localize to detergent-insoluble fractions, particularly caveolae, but clustering has a different role in their trafficking. Even in the absence of clustering, phosphatidylinositol-anchored molecules are detergent-insoluble, which might simply be an intrinsic property of the lipid anchor within the plasma membrane bilayer [10]. Movement of these molecules into caveolae does depend on clustering [47], but clustering in some of these cases might not be physiological [10,47]. In contrast, we have shown that clustering of the transmembrane and cytoplasmic domains of syndecan is required for insolubility in detergent; previous studies have demonstrated several physiologically plausible mechanisms for syndecan clustering (reviewed in [1]), including the binding of multimeric ligands [2]. Thus clustering triggers the syndecan endocytic pathway, and it is reasonable to expect that clustering would occur *in vivo*, which is consistent with a physiological role for syndecan-mediated endocytosis.

Syndecan HSPGs, like other receptors, seem to be organized into domains that mediate specific functions. Portions of the ectodomain, particularly the glycosaminoglycan side chains, serve to bind ligands, whereas those regions within the core protein that contact the cell are responsible for recruiting the specific cellular machinery used for this endocytic pathway. Attractive candidates for mediating the association with detergent-insoluble rafts include the syndecan transmembrane domain, for which indirect evidence already exists [6], and the highly cationic ten initial residues of the cytoplasmic tail [48] (see also [49]). In this light, the distinct pathways for cellular uptake of ligands mediated by the syndecan transmembrane and cytoplasmic domains, by other cell-surface HSPGs [15,50] and by endocytic receptors in coated pits [29,30] can be understood as a consequence of differences in their protein sequences, which lack any significant homology to each other and therefore recruit different cellular machineries for internalization. Because different endocytic pathways are regulated independently (compare Figure 4 with [30]) and often produce distinct metabolic consequences [51–55], the high degree of conservation of syndecans throughout the animal kingdom [56] suggests distinct but essential biological functions for this non-coated-pit endocytotic pathway.

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