Brefeldin A inhibits the endocytosis of plasma-membrane-associated heparan sulphate proteoglycans of cultured rat ovarian granulosa cells

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Rat ovarian granulosa cells were labelled with [^{35}S]sulphate for 0.5–20 h and chased in the presence or absence of 1–2 μg/ml of brefeldin A (BFA) for up to 21 h. Heparan [^{35}S]sulphate (HS) proteoglycans from the culture medium, plasma membrane and intracellular fractions were then analysed by gel chromatography. In the absence of BFA, about 85 % of the plasma membrane-associated HS proteoglycans were endocytosed and subsequently degraded intracellularly. Recirculation of the HS proteoglycans between the intracellular pool and the cell surface was not observed. Exposing the cells to BFA for less than 1 h did not influence the turnover of the HS proteoglycans, whereas the effect of the drug on the Golgi functions reached a maximum in approx. 10 min. When the cells were treated with BFA for more than 1–2 h, the rate of endocytosis of HS proteoglycans was reduced to about 50 % of the control. The delivery of endocytosed HS proteoglycans to lysosomes were not affected by the drug.

Cycloheximide also reduced the endocytosis of HS proteoglycans, but not as much as BFA, indicating that the inhibitory effect of BFA can be only partly accounted for by a block of protein transport from the endoplasmic reticulum to the plasma membrane. In contrast with the endocytosis of HS proteoglycans, neither that of ^{125}I-transferrin, known to be mediated by clathrin-coated vescicles, nor that of ^{125}I-ricin, a marker molecule for bulk endocytosis, was affected by BFA. The half-life of ^{125}I-transferrin and ^{125}I-ricin in the plasma membrane was about 10 and 25 min respectively compared with about 5 h for the HS proteoglycans. Altogether, these results indicate that the endocytosis of plasma-membrane-associated HS proteoglycans is mediated by different mechanisms than the endocytosis of most other cell-surface proteins. Further, the mechanisms involved in the endocytosis of HS proteoglycans are sensitive to BFA.

INTRODUCTION

Most mammalian cells have heparan sulphate (HS) proteoglycans at the cell surface. These proteoglycan molecules are believed to be involved in processes like cell–matrix interactions and binding of various growth factors [1,2]. It has also been shown that cell-surface HS proteoglycans mediate the internalization of basic fibroblast growth factor [3]. The turnover of the plasma-membrane-associated HS proteoglycans has been rigorously studied in cultured rat ovarian granulosa cells [4–9]. These cells synthesize two distinct species of plasma-membrane-intercalated HS proteoglycans: one with intercalated core protein, and the other with glycosyl-phosphatidylinositol (GPI) membrane anchor. These two proteoglycan species can be distinguished by their susceptibility to exogenously added trypsin and phosphatidylinositol-specific phospholipase C (PI-PLC) respectively. The protein-intercalated species is partly shed from the cell surface, but most of it (70 %) is endocytosed and subsequently subjected to a stepwise degradation, generating distinct HS oligosaccharides as degradation intermediates [9]. The GPI-anchored HS proteoglycans are endocytosed, without any shedding into the medium, and thereafter degraded without generating degradation intermediates [9]. The exact mechanisms involved in the endocytosis of the HS proteoglycans have so far not been elucidated. Endocytosis is often considered to be mediated by clathrin-coated vesicles. However, several recent studies clearly indicate the existence of clathrin-independent endocytosis as well

[10–14]. The main subject for the present study was to investigate the endocytosis of cell-surface HS proteoglycans in detail with the use of brefeldin A (BFA).

BFA is a remarkable drug which induces a rapid dissociation of various coat proteins from the membranes of the Golgi apparatus and the *trans*-Golgi network in most mammalian cells [15–17]. The dissociation of the coat proteins from the Golgi membranes results in the disassembly of the *cis*-, *medial*- and *trans*-Golgi subcompartments, followed by a fusion of these subcompartments with the endoplasmic reticulum [18]. BFA has also been shown to have profound effects on endocytotic organelles, inducing the formation of an extensively fused network of membranes derived from the *trans*-Golgi network and early endosomes, as well as the formation of tubular lysosomes [19–21]. Whereas the effect of BFA on the proximal part of the Golgi apparatus results in an inhibition of the transport of macromolecules from endoplasmic reticulum/Golgi apparatus to the *trans*-Golgi network [22–24], the dramatic morphological effects on the endocytotic organelles do not seem to affect the clathrin-mediated endocytic prosess [20,21]. However, BFA has been reported to inhibit transcytosis [25] and to reduce recycling of transferrin receptor [26] in MDCK cells, as well as to impair the transport of molecules from endosomes to lysosomes in various cell types [20].

In the present study we have investigated the effect of BFA on the endocytosis and intracellular degradation of plasma-membrane-associated HS proteoglycans in cultured rat ovarian gran-

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ulosa cells. Our results show that exposure of the cells to BFA for more than 1–2 h was followed by a dramatically reduced rate of endocytosis of the proteoglycan molecules. In contrast, the endocytosis of ^{125}I-transferrin or ^{125}I-ricin were not affected by exposure of the cells to BFA. Transferrin is known to be endocytosed via clathrin-coated vesicles [27]. Ricin, which binds to terminal galactose residues on cell-surface glycoproteins and glycolipids [28–30], has been shown to be endocytosed by both clathrin-dependent and clathrin-independent pathways [11–13]. Since the endocytosis of HS proteoglycans were inhibited by BFA, whereas the endocytosis of ^{125}I-transferrin and ^{125}I-ricin was unaffected, our results may indicate that the endocytosis of the proteoglycan molecules do not follow any of the major endocytic routes. The present study also demonstrates that the transport of HS proteoglycans from endosomes to lysosomes and subsequent lysosomal degradation was not significantly influenced by BFA in the granulosa cells, at least during the first 2 h of exposure to the drug.

EXPERIMENTAL

Materials

$H_2{}^{35}SO_4$ and [³H]leucine were obtained from du Pont–New England Nuclear, Na[125]I and ^{125}I-transferrin from Amersham International. Superose 6 (HR 10/30), Sephadex G-50 (fine) and Q-Sepharose were from Pharmacia LKB Biotechnology; chondroitin ABC lyase (from *Proteus vulgaris*) from Seikagaku America; cycloheximide, trypsin [bovine pancreas, type III, L-1-tosylphenylalanylchloromethane ('TPCK')-treated], transferrin (human, holo form) and pregnant-mare's serum gonadotropin from Sigma; phosphatidylinositol-specific phospholipase C (from *Bacillus thuringiensis*) from American Radiolabelled Chemicals; guanidinium chloride and urea from Life Technologies; BFA from Boehringer Mannheim; Triton X-100 and Iodo-beads from Pierce. Ricin was kindly provided by Dr. Kirsten Sandvig, Institute for Cancer Research at the Norwegian Radium Hospital, Oslo, Norway.

Methods

Cell culture

A total of 5 units of pregnant-mare's serum gonadotropin were injected subcutaneously into 24-day-old female Sprague–Dawley rats. The rats were killed 48 h after injection, and granulosa cells were isolated from the ovaries as previously described [4]. A total of $(3–5) \times 10^6$ cells in 2 ml of medium 199 (M 199), containing 10% fetal bovine serum, were seeded into 35 mm-diameter dishes and cultured at 37 °C in air/CO_2 (19:1).

Pulse–chase experiments

Cell cultures were radiolabelled for 20 h by adding [³⁵S]sulphate to a final concentration of 50 μCi/ml at the time of cell plating, or they were radiolabelled for 30 min with 200 μCi/ml of [³⁵S]sulphate 20 h after plating. After radiolabelling, the cells were washed six times with M 199 over a period of 30 min to remove free [³⁵S]sulphate. The cells were then chased in the absence or presence of BFA or cycloheximide as described in the text. At the end of the chase period the medium fraction was harvested. Plasma membrane-associated, ³⁵S-labelled proteoglycans were harvested by washing the cells twice with serum-free M 199 and subsequently incubating them for 15 min at 37 °C in the presence of 10 μg/ml of trypsin [5]. In some experiments GPI-anchored and protein-intercalated molecules were harvested separately. In these experiments, the cultures were incubated in

the presence of 0.4 unit/ml PI-PLC for 30 min at 37 °C prior to the trypsin treatment. After the removal of incubation medium, the intracellular ³⁵S-labelled macromolecules were solubilized in 4 M guanidinium chloride containing 2% Triton X-100. In initial experiments, different concentrations of BFA were used. Maximal inhibitory effect was obtained with 1 μg/ml. This concentration was therefore used in the following experiments unless otherwise stated in the text.

Analysis of ³⁵S-labelled HS proteoglycans

Unincorporated [³⁵S]sulphate and guanidinium chloride were removed from the samples by gel filtration on Sephadex G-50 (fine) columns [bed volume 4 ml, equilibrated with 0.1 M Tris/0.1 M acetate (pH 7.3)/0.5% Triton X-100] as previously described [31]. The samples were then treated with chondroitin ABC lyase (0.1 unit/ml) to remove chondroitin/dermatan sulphate proteoglycans, and analysed by gel chromatography using Sephadex G-50 (fine) or Superose 6 (HR 10/30) as described below.

Iodination of ricin

Iodo-beads and 0.5 μCi of Na[125]I were added to 25 μg of ricin in 200 μl of PBS, pH 7.4. The solution was incubated at room temperature for 30 min before the reaction was stopped by the addition of 5 μl of 0.1 M KI and 10 μl of 0.1 M $Na_2S_2O_5$. Free Na[125]I was then removed by putting the solution on to a column of Sephadex G-50, which was eluted with PBS containing 0.1% BSA.

Endocytosis of ^{125}I-ricin

The granulosa cells were preincubated with 2 μg/ml BFA in M 199 containing 10% fetal-bovine serum for up to 6 h before the cells were washed three times with serum-free M 199. ^{125}I-Ricin $[(1–3) \times 10^5$ c.p.m.] in 0.6 ml of M 199 was then added to each culture before the cells were transferred to the incubator. After 20 min at 37 °C the cells were washed seven times with ice-cold M 199 to remove unbound ligand. To harvest non-internalized ^{125}I-ricin from the cell surface, the cultures were incubated for 5 min at 37 °C in M 199 containing 0.1 M lactose and subsequently washed three times with the same medium [32]. Alternatively, the cultures were washed three times for 15 min each with ice-cold 0.1 M lactose. In cultures preincubated with BFA, BFA was also present during the wash and the entire chase period. The cells were then solubilized in 4 M guanidinium chloride containing 2% Triton X-100. Endocytosis was measured as the percentage of cell-associated ^{125}I-ricin that could not be removed by lactose.

Endocytosis of ^{125}I-transferrin
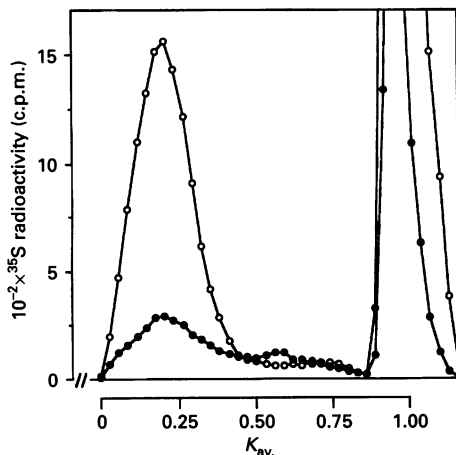
The granulosa cells were preincubated with 2 μg/ml BFA for up to 6 h. At 30 min before the end of the preincubation period the medium was replaced by serum-free M 199 to remove any serum-derived transferrin. At the end of the preincubation period the cells were placed on ice and washed twice with ice-cold 0.15 M NaCl, containing 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.3% (w/v) BSA and 20 mM Hepes, pH 7.2. The cells were then incubated in the presence of ^{125}I-transferrin (10^5 c.p.m.) in the same buffer as described above for 30 min at 4 °C, before the cells were washed five times with ice-cold buffer to remove unbound ligand. Half of the cultures were then washed twice with the same NaCl buffer as above, but with the pH adjusted to 2.0 to remove non-internalized ^{125}I-transferrin from the cell surface [33]. Intra-

cellular ^{125}I-transferrin was then solubilized by adding 4 M guanidinium chloride, containing 2% Triton X-100. The other half of the cultures were incubated for 15 min at 37 °C in M 199, containing 0.3% BSA. The excreted (recycled) ^{125}I-transferrin were then harvested from the medium fractions, before the non-internalized ligand was harvested by washing the cells twice with the low-pH buffer [33]. Finally, the intracellular ^{125}I-transferrin was solubilized by adding 4 M guanidinium chloride/2% Triton X-100. Non-specific binding of ^{125}I-transferrin, as investigated in the presence of 1 mg/ml non-radioactive transferrin, was always less than 10%. In the cultures preincubated with BFA, BFA was also present during the wash and the entire chase period.

RESULTS

Expression of HS proteoglycans in rat ovarian granulosa cells

Rat ovarian granulosa cells were isolated and the metabolism of HS proteoglycans was studied by a series of radiolabelling experiments. After a 30 min pulse with [35S]sulphate, 35S-labelled macromolecules in the plasma membrane and the intracellular compartments were harvested, and unincorporated radioactive precursor was removed by gel filtration. Chondroitin sulphate and dermatan sulphate proteoglycans in both fractions were digested by chondroitin ABC lyase treatment. More than 95% of the chondroitin ABC lyase-resistant 35S-labelled macromolecules could be depolymerized by heparitinase or HNO_2 treatment (results not shown). Consequently, these 35S-labelled macromolecules were almost exclusively of HS nature. Subsequently to the chondroitin ABC lyase treatment, the 35S-labelled macromolecules from both the plasma membrane and the intracellular fraction were analysed by Superose 6 gel chromatography. As shown in Figure 1, about 80% of the cell-associated HS proteoglycans ($K_{av.} \approx 0.2$) were located in the plasma membrane at the end of the 30 min pulse-labelling period, whereas about 20% were located intracellularly. The intracellular fraction also contained very small amounts of HS degradation products, which were eluted at a $K_{av.}$ of 0.5–0.8 [5,6]. The peaks eluted at $K_{av.}$ of 1.0 represent disaccharides derived from depolymerized chondroitin sulphate proteoglycans by the chon-
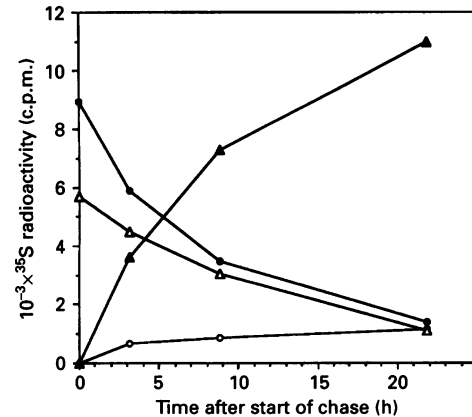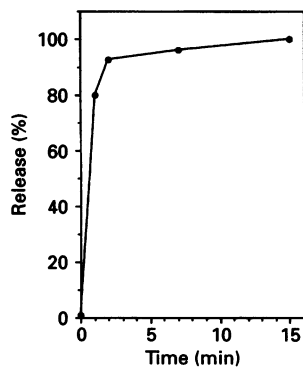

Figure 2 Turnover of HS proteoglycans

Rat ovarian granulosa cells were labelled with [35S]sulphate for 20 h and then chased for up to 21 h. After 0, 3, 8.5, and 21 h the culture medium, plasma membrane (trypsin-releasable), and intracellular (trypsin-resistant) fractions were harvested and the amount of 35S-labelled HS proteoglycans determined after chondroitin ABC lyase treatment and Superose 6 gel chromatography. Each data point represents the average of duplicate determinations. The S.D. was less than 15% at all data points. ●, Plasma-membrane fraction; ○, medium fraction; △, intracellular fraction; ▲, free [35S]sulphate.

droitin ABC lyase treatment. In some experiments the plasma-membrane fraction was separated into a GPI-anchored and a protein-intercalated fraction. These experiments showed that about 30% of the cell-surface HS proteoglycans was of the GPI type, whereas 70% was of the core-protein-intercalated type (results not shown).

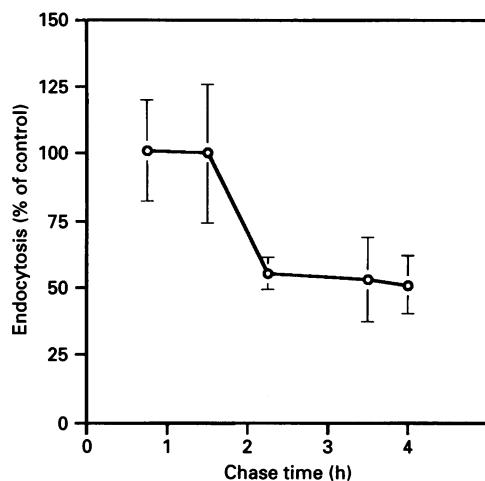### Endocytosis of plasma-membrane-associated HS proteoglycans

To study the endocytosis of the cell-surface HS proteoglycans, the cells were pulse-labelled with [35S]sulphate for 20 h and then chased for up to 21 h after removal of the labelling precursor. At the end of the different chase periods, the culture medium, the plasma membrane and the intracellular fractions were harvested and the amount of 35S-labelled HS proteoglycans in each fraction was determined by gel chromatography after chondroitin ABC lyase treatment. It was found that about 85% of the cell-surface HS proteoglycans were endocytosed and subsequently degraded intracellularly, whereas the rest was shed to the culture medium (see Figure 2, in which the results from one typical experiment are shown). By comparing the amount of 35S-labelled HS proteoglycans in the culture medium, the plasma membrane and the intracellular fraction after various chase times, the rate of endocytosis can be calculated. Our experiments revealed that the cell-surface 35S-labelled HS proteoglycans had a half-life of about 5 h in the plasma membrane (as shown in Figure 2), results almost identical with those previously reported [9]. The reduction in the intracellular pool of 35S-labelled HS and the liberation of free [35S]sulphate shown in Figure 2, reflect intracellular degradation of HS proteoglycans and will be discussed in a separate section below ('Effect of BFA on intracellular degradation of HS proteoglycans').

To investigate whether the apparently slow turnover ($t_{\frac{1}{2}} \approx$ 5 h) of the cell-surface HS proteoglycans was due to recirculation back to the plasma membrane of endocytosed HS molecules, experiments were performed in which the cells were pulse-labelled with [35S]sulphate for 20 h and then chased in the presence of trypsin for 1–15 min. The amount of 35S-labelled HS

Figure 1 Expression of HS proteoglycans

Rat ovarian granulosa cells were labelled with [35S]sulphate for 30 min. 35S-labelled macromolecules from the plasma membrane (trypsin-releasable) and intracellular compartments (trypsin-resistant) were then isolated and analysed by Superose 6 gel chromatography after chondroitin ABC lyase treatment. ○, Plasma-membrane fraction; ●, intracellular fraction.

Figure 3   Absence of HS proteoglycan recirculation
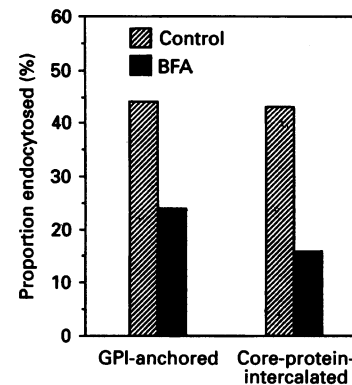
Rat ovarian granulosa cells were labelled with [35S]sulphate for 20 h before the cells were chased in serum-free medium containing 50 μg/ml trypsin. After 1, 2, 7 and 15 min the trypsin-releasable fraction was harvested and analysed by Superose 6 gel chromatography after chondroitin ABC lyase treatment. The amount of 35S-labelled HS proteoglycans in the trypsin-releasable fraction at each time point is expressed as a percentage of the amount of 35S-labelled HS proteoglycans present after 15 min. Each data point represents the average of duplicate determinations. The S.D. was less than 10% for all time points.

Figure 4   Effect of BFA on the endocytosis of cell-surface HS proteoglycans

Rat ovarian granulosa cells were labelled with [35S]sulphate in the absence of 1 μg/ml BFA for 20 h and then chased in the absence or presence of BFA for various periods. At the start and the end of the chase periods the culture medium, plasma-membrane (trypsin-releasable) and intracellular (trypsin-resistant) fractions were harvested and analysed by Superose 6 gel chromatography after chondroitin ABC lyase treatment. The amount of 35S-labelled HS proteoglycans in each fraction was determined and used to calculate the amount of 35S-labelled HS proteoglycans endocytosed during the chase period. The data points, which represent mean values $\pm$ S.D., express the endocytosis of HS proteoglycans in BFA-treated cultures as a percentage of the endocytosis in control cultures. Values are based on the results from five separate experiments.

proteoglycans in the trypsin-releasable (cell-surface) fraction at each time point was expressed as percentage of the total plasma-membrane fraction (the amount of 35S-labelled HS proteoglycans in the trypsin-releasable fraction after 15 min). Since the amount of HS proteoglycans released by 2 and 15 min trypsin treatment was virtually the same (Figure 3), any significant recirculation of endocytosed HS proteoglycans back to the cell surface taking longer than 2 min can be excluded. Although cycling between cell-surface and intracellular compartments may occur in less than

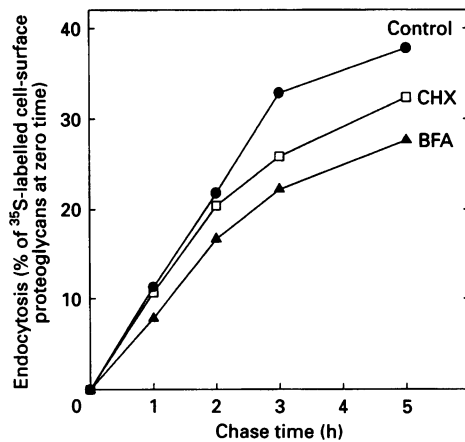## Figure 5   Endocytosis of GPI-anchored and protein-intercalated HS proteoglycans

Rat ovarian granulosa cells were labelled with [35S]sulphate for 20 h before the cells were chased in the absence or presence of 1 μg/ml BFA for 6 h. At the start and the end of the chase period the culture medium, PI-PLC-releasable, trypsin-releasable, and intracellular (trypsin-resistant) fractions were analysed by Superose 6 gel chromatography after chondroitin ABC lyase treatment. The amount of 35S-labelled HS proteoglycans in each fraction was determined and used to calculate the amount of GPI-anchored and protein-intercalated 35S-labelled HS proteoglycans endocytosed during the chase period. The bars represent the percentages of GPI-anchored and protein-intercalated 35S-labelled HS proteoglycans present at the cell surface at the start of the chase period that had been endocytosed after 6 h. The values are based on averages from two separate experiments, each done in triplicate. The S.D. was less than 20% for all values.

2 min, previous experiments using timed trypsin treatment, as well as repeated challenges by trypsin, indicate that no measurable cycling HS proteoglycan pool exists in the granulosa cells [6].

Effect of BFA on the endocytosis of HS proteoglycans

To study the effect of BFA on the endocytosis of the cell-surface HS proteoglycans, the cells were pulse-labelled with [35S]sulphate for 20 h in the absence of BFA and then chased in the absence or presence of BFA for up to 21 h after the removal of the labelling precursor. The rate of endocytosis was calculated as described above. These experiments showed that BFA had no immediate effect on the endocytosis of cell-surface HS proteoglycans (Figure 4), whereas the effect of the drug inhibiting normal Golgi functions reached a maximum within 10 min in the same cell system [35]. However, after 1–2 h of exposure to BFA, the endocytosis of 35S-labelled HS proteoglycans was reduced to about 50% (mean for five separate experiments) of the control (Figure 4). Although the onset of the inhibitory effect of BFA varied somewhat from experiment to experiment, the effect was relatively constant when the cells were chased in the presence of BFA for more than 2 h. BFA did not significantly affect the shedding of 35S-labelled HS proteoglycans to the culture medium (results not shown).

Since the intracellular processing after endocytosis is distinct for GPI-anchored and protein-intercalated HS proteoglycans, experiments were performed to study the effect of BFA on the endocytosis of these two species of proteoglycans. In these experiments the cells were labelled with [35S]sulphate for 20 h and then chased in the absence or presence of BFA for 6 h after removal of the labelling precursor. The cells were then sequentially treated with PI-PLC and trypsin to quantify GPI-anchored and protein-intercalated HS proteoglycans respectively. These experiments revealed that the endocytosis of both species of proteoglycans were inhibited by BFA (Figure 5). It

Figure 6   Effect of cycloheximide on the endocytosis of HS proteoglycans

Rat ovarian granulosa cells were labelled with [^{35}S]sulphate for 20 h before the cells were washed and chased for the indicated times in the absence (control) or presence of 50 μg/ml cycloheximide (CHX) or 1 μg/ml BFA. Endocytosis was calculated as described in the legend to Figure 4. Averages of determinations at each time point (three data points for control, two for the BFA and cycloheximide groups) were used for the calculation. The average coefficient of variation of all determinations was 4%.

may be noteworthy that some differences in the extent of BFA effect were observed for the GPI-anchored and the protein-intercalated HS proteoglycans (Figure 5), which may reflect differences in their endocytotic mechanisms.

Effect of cycloheximide on the endocytosis of HS proteoglycans

Different types of endocytosis involve various plasma-membrane-associated proteins. The turnover of most of these proteins seems to be relatively slow, since endocytosis of various ligands is known to continue for several hours after cycloheximide-induced block of protein synthesis. BFA blocks the anterograde transport of proteins from the endoplasmic reticulum in most mammalian cells [22,23,34], including rat ovarian granulosa cells [35]. Since our results showed that the inhibitory effect of BFA on the endocytosis of HS proteoglycans was seen only after prolonged (longer than 1–2 h) exposure to the drug, the inhibitory effect of BFA may be due to depletion of one or more plasma-membrane-associated protein(s) involved in the endocytotic process by the blocked transport of proteins. (Protein synthesis of granulosa cells *per se* is relatively unaffected by BFA. Levels of the total protein synthesis measured by [^{3}H]leucine incorporation after BFA treatment were $\approx$ 100 % and 85 % of the untreated control at 30 min and 4 h respectively.) Experiments were therefore performed to explore whether exposure of the cells to cyclo-heximide also inhibited the endocytosis of HS proteoglycans. In these experiments the cells were pulse-labelled with [^{35}S]sulphate for 20 h before they were chased for 1–5 h in the presence of cycloheximide or BFA. These results showed that exposure of the cells to cycloheximide for more than 2 h resulted in a 27 % reduction in the rate of endocytosis of HS proteoglycans, compared with the control (average from two separate experiments). However, the inhibitory effect of cycloheximide was significantly less (about 50 %) compared with the inhibitory effect of BFA. The results from one typical experiment are shown in Figure 6.

Reversible effect of BFA on the endocytosis of HS proteoglycans

The inhibitory effect of BFA on the transport and secretion of proteins is reported to be fully reversible after removal of the drug [22]. To investigate whether the inhibitory effect of BFA on the endocytosis of HS proteoglycans is also reversible, [^{35}S]sulphate-labelled cultures were first chased in the presence or absence of BFA for 4 h. The cells were then washed; BFA was removed from some cultures before all cultures were chased for another 2 h in the presence or absence of BFA. As expected, in the cultures chased in the presence of BFA for the total of 6 h, the endocytosis of HS proteoglycans was inhibited about 50 %. However, in the cultures chased in the presence of BFA for the first 4 h, and then in the absence of BFA, the rate of endocytosis was at the level of the control cultures 2 h after removal of the drug, clearly indicating that the inhibitory effect of BFA on endocytosis of HS proteoglycans is reversible (results not shown).

## Effect of BFA on the endocytosis of ^{125}I-transferrin

Most mammalian cells have transferrin receptors in their plasma membrane. When transferrin is bound to the receptor, it is endocytosed via clathrin-coated vesicles, usually within a few minutes. The endocytosis of transferrin is therefore often used as a marker for clathrin-mediated endocytosis. To investigate if BFA had any inhibitory effect on endocytosis mediated by clathrin-coated vesicles, ^{125}I-transferrin was added to granulosa cells preincubated with BFA, and the endocytosis of the ^{125}I-transferrin was then studied as described under 'Methods'. These experiments revealed that preincubation of the cells with BFA for up to 6 h had no significant inhibitory effect on the endocytosis of ^{125}I-transferrin (results not shown). Further, the experiments showed that the ^{125}I-transferrin was internalized much more rapidly ($t_{\frac{1}{2}} \approx$ 10 min) than the ^{35}S-labelled HS proteo-glycans ($t_{\frac{1}{2}} \approx$ 5 h).

Effect of BFA on the endocytosis of ^{125}I-ricin

Ricin is known to bind to terminal galactose residues of plasma-membrane glycoproteins and glycolipids [28–31], and can therefore be used as a marker for endocytosis of such molecules. It has also been shown that the endocytosis of ricin can take place in cells in which the clathrin-mediated endocytosis is blocked, indicating that ricin, at least partly, is internalized via clathrin-independent mechanisms [11–13]. To study the effect of BFA on the endocytosis of ricin-binding molecules, as well as clathrin-independent endocytosis, ^{125}I-ricin was added to granulosa cells preincubated with BFA. The endocytosis of the ^{125}I-ricin was then investigated as described in 'Methods'. The results clearly showed that pretreatment of the granulosa cells with BFA for up to 6 h did not influence the internalization of the ^{125}I-ricin (results not shown). Like ^{125}I-transferrin, the ^{125}I-ricin was rapidly endocytosed ($t_{\frac{1}{2}} \approx$ 25 min), indicating that ricin-binding plasma membrane glycoproteins/glycolipids have a mean half-life in the plasma membrane of the granulosa cells of about 25 min (in contrast with about 5 h for the cell-surface HS proteoglycans).

Effect of BFA on intracellular degradation of HS proteoglycans
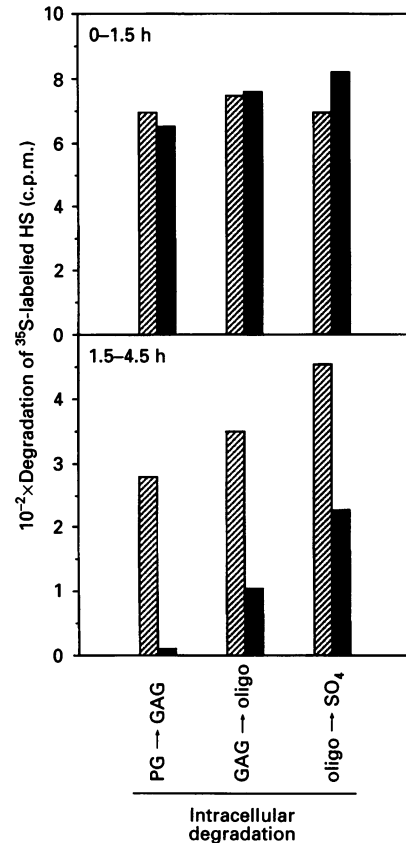
Previous studies have shown that, in cultured granulosa cells, endocytosed plasma-membrane HS proteoglycans are subjected to intracellular degradation [9]. The degradation of the proteo-glycan molecules is a stepwise process, first generating free
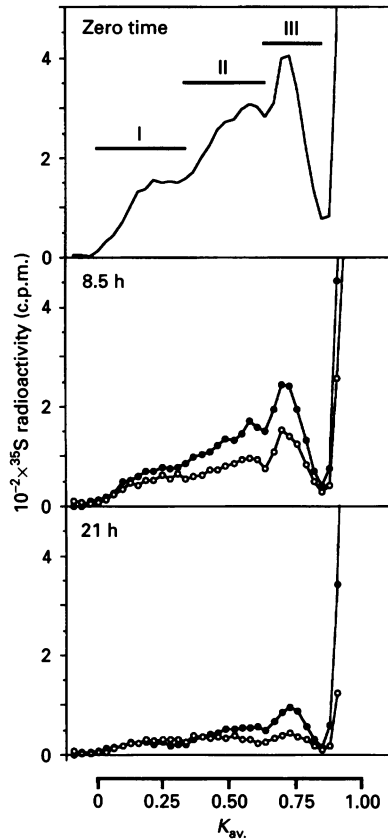
Figure 7   Superose 6 gel chromatography of intracellular fractions from the pulse–chase experiment

Rat ovarian granulosa cells were labelled with [^{35}S]sulphate for 20 h and then chased for in the absence or presence of BFA. At the start of the chase (upper panel) and after 8.5 h (centre panel) and 21 h (lower panel), the intracellular fraction was harvested and analysed by Superose 6 gel chromatography after treatment with chondroitin ABC lyase. ●, Control; ○, BFA-treated.

Figure 8   Rate of intracellular degradation

Rat ovarian granulosa cells were labelled with [^{35}S]sulphate for 20 h and then chased in the absence or presence of BFA. At the start of the chase, and after 1.5 and 4.5 h, the culture medium, plasma-membrane and intracellular fractions were harvested and analysed by gel chromatography after chondroitin ABC lyase treatment. The amount of ^{35}S-labelled HS proteoglycans endocytosed, as well as the intracellular amount of intact ^{35}S-labelled HS proteoglycan ($K_{av.} \approx 0.20$ on Superose 6), HS glycosaminoglycan (GAG) chains ($K_{av.} \approx 0.5$) and HS oligosaccharides (oligo) ($K_{av.} \approx 0.75$), was then determined for each time point and used to calculate the rate of degradation from proteoglycan (PG) to GAG, from GAG to oligosaccharides (oligo) and from oligosaccharides to free [^{35}S]sulphate (SO$_4$), during the first 1.5 h (upper panel) and the last 3 h (lower panel) of the chase period. Averages of determinations were used for the calculation. The S.D. was less than 15% for all values. Cross-hatched columns, control; solid columns, BFA-treated.

glycosaminoglycan chains, then partly degraded glycosaminoglycan chains, and finally, monosaccharides and free sulphate. The degradation appears to take place both in pre-lysosomal and lysosomal compartments [6]. Hence the generation of HS degradation products may be used as a marker for intracellular transport from early endosomes to lysosomes. To study the effect of BFA on the degradation of endocytosed HS proteoglycans, and their transport to the lysosomes, the cells were pulse-labelled with [^{35}S]sulphate for 20 h and then chased for up to 21 h in the absence or presence of BFA. The intracellular fractions were analysed by gel chromatography after chondroitin ABC lyase treatment. As shown in Figure 7, which shows the results from one typical experiment, the chondroitin ABC lyase-resistant material was eluted in three peaks (as indicated in upper panel); peak I ($K_{av.} \approx 0.20$) represents intact HS proteoglycans, peak II ($K_{av.} \approx 0.5$) represents free HS chains, whereas peak III ($K_{av.} \approx 0.75$) represents HS oligosaccharides generated from the glycosaminoglycan chains. ^{35}S-labelled HS molecules from both control (●) and BFA cultures (○) are displayed in the centre (8.5 h chase) and lower (21 h chase) panels. The profiles for both the control and the BFA are almost identical, indicating that intracellular degradation, and hence, transport to the lysosomes, of HS proteoglycans also takes place when the cells were chased in the presence of BFA for up to 21 h. By comparing the amount of ^{35}S-labelled HS molecules in each peak (I–III) at various chase

times, as well as the amount of ^{35}S-labelled HS proteoglycans endocytosed, the rate of degradation could be calculated. The result of calculation, as summarized in Figure 8, indicates that BFA did not have any immediate effect on the degradation of ^{35}S-labelled HS proteoglycans. However, 1.5 h after the addition of BFA, the total amount of ^{35}S-labelled HS degraded were significally reduced at all three steps in the degradation process. The reduced degradation may be due to an inhibitory effect of BFA on the transport of ^{35}S-labelled HS to the lysosomes, but may also be due to the reduced endocytosis of HS proteoglycans seen after 1–2 h of BFA treatment, since reduced endocytosis of ^{35}S-labelled HS proteoglycans will be followed by a reduction in the amount of ^{35}S-labelled HS proteoglycans available for degradation. However, it is noteworthy that, in three separate experiments (and as shown in Figure 8), the first step in the degradation process (the generation of free glycosaminoglycan chains) was more reduced in the presence of BFA than the subsequent degradation steps. This may reflect reduced HS

proteoglycan endocytosis or a specific inhibitory effect of BFA on the first step in the degradation of HS proteoglycans.

DISCUSSION

Most mammalian cells have HS proteoglycans associated with the plasma membrane. These proteoglycans may be linked to the cell surface via a GPI anchor or by a core protein intercalated in the plasma membrane. Rat ovarian granulosa cells synthesize both these species of proteoglycans. In the granulosa cells both the GPI-anchored and the protein-intercalated HS proteoglycans are turned over mainly by endocytosis and subsequent intracellular degradation [9]. The results presented here demonstrate that both species of HS proteoglycans are internalized about 30 times slower than transferrin and about 10–15 times slower than ricin. The slow turnover of the HS proteoglycans may fit well with the hypothesis that these molecules participate in relatively stable processes like cell attachment and accumulation of growth factors at the cell surface. Similarly, integrins, another group of cell-surface molecules involved in cell attachment, are reported to be internalized at a rate at least 10 times slower than that of the transferrin receptor [36]. The low rate of endocytosis of HS proteoglycans may indicate that these molecules are excluded from coated-pit structures. Clustering and endocytosis in clathrin-coated pits is thought to require signals in the cytoplasmic domain of transmembrane proteins [37,38]. In fact, GPI-anchored proteins, which lack such signals, are believed to be associated with non-clathrin-coated invaginations or caveolae [39], from where they may be released by GPI-specific phospholipase D [40], or internalized by non-clathrin-mediated mechanisms [41]. Also some transmembrane proteins have been reported to be excluded from coated pits. The mechanisms of exclusion from coated pits of such molecules are not known, but it has been suggested that interaction with protein tyrosine kinase p56^{lck} [42] or with cytoskeletal elements [43] play a vital role.

In order to study the mechanisms involved in the endocytosis of cell-surface HS proteoglycans in more detail, we have used BFA as an experimental tool. Although BFA is reported to induce profound effects on endocytotic organelles [19–21], it has not been shown to influence the rate of endocytosis of any molecule. The results presented here have also demonstrated that BFA had no immediate effect on the endocytosis of plasma-membrane-associated HS proteoglycans. However, after 1–2 h of BFA treatment, the internalization of HS proteoglycans was dramatically reduced. BFA has been reported to induce dissociation of coat proteins from the Golgi membranes, leading to a disassembly of the Golgi apparatus within minutes after addition of the drug to cell cultures [44]. Effects of BFA on several Golgi functions in granulosa cells also follow very similar kinetics [35]. If the inhibitory effect of BFA on the endocytosis of HS proteoglycans in the granulosa cells was due to a dissociation of coat proteins from the plasma membrane, the effect of BFA on the endocytosis of HS proteoglycans should have been observed earlier than 1–2 h after the addition of the drug to the cells. Therefore, a more likely explanation for the inhibitory effect of BFA may be that one or more plasma-membrane component(s) necessary for internalization of HS proteoglycans have a relatively rapid turnover, and they are depleted by the block in anterograde transport from the endoplasmatic reticulum induced by BFA. This hypothesis was partly confirmed by the cycloheximide experiments, which showed that a block in protein synthesis was followed by a reduced endocytosis of ^{35}S-labelled HS proteoglycans about 2 h after the addition of cycloheximide to the cell cultures. However, the cycloheximide-induced re-

duction in endocytosis was only about 50 % of the BFA-induced reduction, indicating that BFA also had a direct inhibitory effect on the endocytosis of HS proteoglycans.

The endocytosis of both GPI-anchored and protein-intercalated HS proteoglycans was both inhibited by BFA. In contrast, we found that the internalization of ^{125}I-transferrin and ^{125}I-ricin was not influenced by pretreatment of the cells with BFA up to 6 h, in agreement with reports by other investigators [20,45]. Since transferrin is a marker for clathrin-mediated endocytosis, and ricin binds to various cell-surface glycoproteins/glycolipids endocytosed by both clathrin-dependent and clathrin-independent mechanisms, our results indicate that the endocytosis of plasma-membrane-associated HS proteoglycans do not follow any of the major endocytic routes. The long half-life of the HS proteoglycans at the cell surface compared with the ricin-binding cell-surface glycoproteins/glycolipids, may indicate that the proteoglycans are actively excluded from the bulk internalization flow. If that is the case, the inhibitory effect of BFA may be due to interference with the mechanisms which ultimately induce endocytosis of the proteoglycan molecules.

It has been speculated that the morphological changes in endocytic organelles induced by BFA are followed by disturbances in the transport from endosomes to lysosomes. In fact, it has been reported that BFA impairs the transport from endosomes to lysosomes in various cells [20]. In the present paper we demonstrate that BFA does not influence the degradation process of HS proteoglycans in granulosa cells, at least during the first 1–2 h of exposure to the drug. Since the last steps in the degradation process of HS is supposed to take place in the lysosomes, our results indicate that exposure of the granulosa cells to BFA has no immediate effect on the transport of HS molecules to the lysosomes. Our results also show that, when the granulosa cells were exposed to BFA for more than 1–2 h, lesser amounts of HS proteoglycans were subjected to degradation compared with the control. The reduced degradation of HS proteoglycans after prolonged exposure of the cells to BFA may be due to reduced transport from early endosomes to lysosomes. However, the lower rate of degradation of HS proteoglycans may also be due to reduced internalization, since reduced endocytosis of HS proteoglycans will give less HS proteoglycans available for degradation.

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