Basic fibroblast growth factor does not prevent heparan sulphate proteoglycan catabolism in intact cells, but it alters the distribution of the glycosaminoglycan degradation products

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Heparan sulphate proteoglycans on cell surfaces have been shown to mediate the degradation or recycling of several ligands. Since the interaction with ligand may affect proteoglycan catabolism once the complex is internalized, this could alter the cellular pool of heparan sulphate chains, with possible consequences for heparan sulphate-mediated cellular processes. We have recently demonstrated that the specific binding of basic fibroblast growth factor (bFGF) to heparan sulphate chains prevents the glycosaminoglycan from being degraded by partially purified heparanases from Chinese hamster ovary (CHO) cells [Tumova and Bame (1997) J. Biol. Chem. **272**, 9078–9085]. The present study examines the effect of bFGF on heparan sulphate catabolism in intact cells. The distribution and size of the heparan sulphate degradation products in CHO cells was analysed in the presence and absence of bFGF using pulse–chase protocols. Although heparan sulphate molecules and bFGF are internalized through the same pathway, even relatively high concentrations of the growth factor do not have any inhibitory effects on glycosaminoglycan degradation. However, the interaction with the growth factor alters the distribution of heparan sulphate-degradation products, presumably by preventing secretion of the short heparanase-derived species. Our findings show that most of the free and bFGF-bound heparan sulphate chains are destined for lysosomes, which would be consistent with a recent hypothesis that the primary role of proteoglycan-mediated internalization of the growth factor is to remove bFGF from its site of action at the cell surface. However, in the presence of bFGF, a fraction of intracellular, heparanase-degraded heparan sulphate chains is delivered to the nucleus, suggesting that the glycosaminoglycan accompanies the growth factor to the organelle. It may be important for bFGF activity that the growth factor is protected from proteolytic degradation by its interaction with heparan sulphate. This work demonstrates that the internalization of a ligand along with the proteoglycan can affect the sorting of heparan sulphate-degradation products in endosomes, and the ultimate destination of the short glycosaminoglycan. It also provides evidence that formation of heparan sulphate–ligand complexes may regulate the recycling and degradation of both ligands and heparan sulphate chains and, consequently, affect their biological activities.

Key words: Endosome, heparanase, intracellular sorting, nuclear targeting, secretion.

INTRODUCTION

For many years, heparan sulphate proteoglycans were considered to be merely structural components involved in extracellular matrix organization and cell–cell or cell–matrix attachment [1,2]. Later, numerous studies demonstrated that the glycosaminoglycan part of the molecule can bind a variety of ligands and, in some instances, these interactions are crucial for ligand activity, stability and metabolism [3,4]. Some ligands, such as lipoprotein lipase [5], basic fibroblast growth factor (bFGF) [3] and γ-interferon [6], require heparan sulphate fragments of a specific length and with characteristic modifications for an optimal interaction. This suggests that both the modification process during heparan sulphate synthesis and the degradation of the glycosaminoglycan by heparanases will play important roles in regulating the ability of heparan sulphate to interact with ligands. Some of the heparan sulphate-binding ligands are internalized with heparan sulphate proteoglycans [7–10], implicating proteoglycan catabolism in controlling both ligand and heparan sulphate availability. If the interaction with a ligand interferes with glycosaminoglycan degradation, it might generate a specific population of heparan sulphate chains, or alter the destination of the degradation products.

In this study, the interaction of heparan sulphate with bFGF was used as a model system to examine the catabolic fate of heparan sulphate proteoglycans when they act as receptors. bFGF is a potent angiogenic growth factor [11,12] that requires heparin or heparan sulphate for its biological activity mediated through tyrosine kinase signalling [13–15]. It binds with relatively high affinity to a specific heparan sulphate sequence that lies within a sulphated, iduronate-rich domain [3]. bFGF was shown to be internalized while bound to heparan sulphate proteoglycans in Chinese hamster ovary (CHO) cells, fibroblasts and epithelial cells [10,16]; however, the fate of the glycosaminoglycans complexed to the growth factor was not investigated. The interaction with heparan sulphate glycosaminoglycans protects bFGF from proteolytic degradation [17,18] and, unlike other internalized growth factors, bFGF remains inside the cells in an active form for several hours [19]. Since our previous studies *in vitro* demonstrated that in this complex heparan sulphate can also be

Abbreviations used: bFGF, basic fibroblast growth factor; CHO, Chinese hamster ovary.

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protected from being cleaved by partially purified heparanases [20], it was of interest to determine whether glycosaminoglycan catabolism in intact cells would be affected in the presence of the growth factor. Because of the high bFGF concentrations required to inhibit heparan sulphate chain degradation *in vitro*, it is unlikely that the interaction with the growth factor would cause dramatic changes in the amount of short chains created by heparanases inside CHO cells. However, since the heparanase-derived heparan sulphate oligosaccharides are still able to bind bFGF with similar affinity as the nascent chains, it is possible that the interaction with the growth factor would alter the destination of the short heparan sulphate products.

To investigate the effect of bFGF on the fate of heparan sulphate chains, glycosaminoglycan degradation in CHO cells was followed in the presence and absence of bFGF. Both the size and the localization of ^{35}S-labelled heparan sulphate-degradation products was determined. In addition, since bFGF was reported to translocate into the nucleus [19,21], CHO cell nuclei were examined for the presence of bFGF and heparan sulphate chains to establish whether the growth factor is delivered to its nuclear destination in a complex with the glycosaminoglycan. CHO cells do not express detectable amounts of the FGF receptors [13,22], and therefore provide an ideal system to study direct effects of bFGF on proteoglycan catabolism without interference from receptor-mediated tyrosine kinase signalling.

MATERIALS AND METHODS

Cell culture

CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Rockville, MD, U.S.A.) and cultured as described previously [23]. All radiolabelling experiments were performed with sulphate-free defined medium with 10 mM glucose [24].

Time course of heparan sulphate catabolism

Nearly confluent CHO cells in 35-mm plates were incubated for 1 h in defined F-12 media containing 50 μCi/ml [^{35}S]H_2SO_4 (NEN Life Sciences Products, Boston, MA, U.S.A.) [23]. The labelling media were removed, and the cells were washed 3 times with PBS and incubated in F-12 media containing 1 mM Na_2SO_4 and 0, 0.5 ng/ml or 2.5 μg/ml bFGF (Promega, Madison, WI, U.S.A.) for 1 h at 4 °C. The cells were then shifted to 37 °C and, at various times (0–12 h), the chase media were removed, cells were washed 3 times with PBS, and the ^{35}S-labelled glycosaminoglycans were isolated from both media and cells [23]. Briefly, the glycosaminoglycans were purified on DEAE–Sephacel columns (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) and precipitated with 80% (v/v) ethanol after the addition of 0.5 mg/ml chondroitin sulphate carrier as described previously [23]. Residual peptides were removed from the glycosaminoglycans by incubating them with 0.5 M NaOH/1 M $NaBH_4$ at 4 °C for 16 h. After neutralization with 10 M acetic acid, the glycosaminoglycans were reprecipitated with ethanol, dried and incubated with 100 m-units of chondroitin ABC lyase (EC 4.2.2.4; ICN Biochemicals, Costa Mesa, CA, U.S.A.) for 16 h at 37 °C to degrade the chondroitin sulphate. Heparan sulphate chains were separated from chondroitin sulphate disaccharides by DEAE–Sephacel columns, desalted on a PD-10 gel-filtration column (1.5 × 5 cm; Pharmacia LKB Biotechnology) and freeze–dried. The size of the heparan sulphate chains was examined by gel filtration on a TSK 4000 HPLC column (7.5 × 30 mm; TosoHaas, Montgomeryville, PA, U.S.A.) calibrated with heparin, heparan sulphate and chondroitin sulphate polysaccharides of known molecular mass. The column was equilibrated and run in 0.1 M KH_2PO_4 buffer (pH 6.0)/0.5 M NaCl/0.2% *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propansulphonate (Zwittergent), at a flow rate of 0.5 ml/min [23]. Fractions of 0.5 ml were collected and assayed for radioactivity. Nascent heparan sulphate chains from CHO cells are, on average, 81 kDa, and the short degradation products generated by heparanases are 6 kDa.

Endosomal and lysosomal localization of heparan sulphate-degradation products

Confluent CHO cells were incubated for 1 h with [^{35}S]H_2SO_4 (50 μCi/ml of medium) to label the proteoglycans on the cell surface and then chased for various time intervals in non-radioactive medium in the presence or absence of bFGF, as explained above. Alternatively, instead of labelling heparan sulphate, ^{125}I-bFGF (75000–150000 c.p.m.; Amersham Life Science, Arlington Heights, IL, U.S.A.) was added with the unlabelled growth factor to follow bFGF localization. At the end of the incubation intervals, the media were removed from both labelled cells and unlabelled carrier cells (approximately 1×10^8 cells per sample), and the monolayers were washed with cold 10 mM Hepes/0.1 M NaCl/20 mM KCl (pH 7.4). The washed cells were removed from the plates by scraping or trypsinization and pelleted in a clinical centrifuge. After washing the cell pellets twice with the Hepes buffer, the cells were resuspended in 10 mM triethanolamine/10 mM acetate/0.25 M sucrose/1 mM EDTA (pH 7.4). The mixture was incubated for 10 min on ice and then the cells were broken with 15 strokes of a tight glass Dounce homogenizer (type B). The homogenate was centrifuged for 10 min at 750 ***g*** to remove unbroken cells, nuclei and cell debris. The postnuclear supernatant was diluted to 9.3 ml with the 10 mM triethanolamine/10 mM acetate/0.25 M sucrose/1 mM EDTA (pH 7.4) buffer, and 4 ml of 0.5 M sucrose/80% Percoll was added. The mixture was centrifuged in a vertical rotor (NVT 65, Beckman) at 26000 ***g*** for 1 h to separate endosomal and lysosomal material [25]. Fractions of 0.75 ml were collected from the top of the gradient using a fraction-recovery system (ISCO, Lincoln, NE, U.S.A.) and aliquots assayed for radioactivity on a Beckman scintillation counter ([^{35}S]heparan sulphate), or a Beckman gamma-counter (^{125}I-bFGF). Endosomes were localized by short-term endocytosis of FITC–dextran [25], whereas lysosomal material was identified by assaying for acid phosphatase [26] and β-hexosaminidase [27] activity.

To analyse heparan sulphate-degradation products in endosomal and lysosomal populations, the corresponding fractions were pooled, and Triton X-100 was added to a final concentration of 0.5%. Glycosaminoglycan material was purified over DEAE–Sephacel columns and treated with NaOH/$NaBH_4$ to release the chains from protein cores [23]. The glycosaminoglycans were then incubated with chondroitin ABC lyase to degrade [^{35}S]chondroitin sulphate and the resulting chondroitin sulphate disaccharides were separated from the heparan sulphate chains on a DEAE–Sephacel column. The size of heparan sulphate glycosaminoglycans in both compartments was determined by gel filtration on the TSK 4000 column.

Immunofluorescence

Confluent CHO cell monolayers were equilibrated with 2.5 μg/ml bFGF as described above, incubated for 2 h at 37 °C, and harvested by scraping in PBS, pH 7.4. Cells were pelleted, briefly washed with cold PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After a single PBS rinse, cells were rapidly pelleted in 10% gelatin in PBS (37 °C) and

allowed to solidify on ice [28]. The cell-containing gelatin block was cut to approx. 1 mm^3 pieces, equilibrated in 2.07 M sucrose/10 % poly(vinylpyrrolidone)/22 mM Na_2CO_3/0.05 % NaN_3 overnight, and then frozen in liquid nitrogen. Sections (1–2 μm) were cut from the blocks with a Reichert Ultracut S microtome (Leica AG, Reichert Division, Austria), and mounted on glass slides. The sections were incubated with either goat anti-human bFGF (40 μg/ml; R&D Systems, Minneapolis, MN, U.S.A.) or mouse anti-heparan sulphate (200 μg/ml; Seikagaku America Inc., Ijamsville, MD, U.S.A.), or a mixture of the two antibodies, for 90 min at room temperature. Sequential 1 h incubations with biotinylated anti-goat secondary antibodies (Vector Laboratories, Burlingame, CA, U.S.A.) and FITC-conjugated streptavidin (dilution 1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.) were used to detect bFGF. Both the secondary antibody and the FITC–streptavidin were diluted 1:500 in 50 mM Tris/150 mM NaCl (pH 7.4). Rhodamine-conjugated anti-mouse immunoglobulins (dilution 1:50; Jackson ImmunoResearch Laboratories) were used to label heparan sulphate. The sections were photographed using T-Max 400 film (Eastman Kodak Company, Rochester, NY, U.S.A.) on an Olympus BX-50 microscope (Olympus America, Inc., Lake Success, NY, U.S.A.), with appropriate filters for the fluorescent labels. The specificity of the antibodies was determined by this immunofluorescence procedure. Cells incubated without bFGF were used as the negative control for the anti-bFGF immunoglobulins. As the negative control for the anti-heparan sulphate immunoglobulins, thin sections were treated for 1 h at 37 °C with 5 m-units/ml heparitinase I (Seikagaku America) in 0.1 M NaCl/50 mM sodium acetate (pH 7.0)/100 μg/ml BSA.

Nuclear localization of heparan sulphate and bFGF

CHO cells, grown to confluence in 35-mm plates, were incubated for 1 h at 4 °C with 75000–150000 c.p.m. of ^{125}I-bFGF in the presence of various concentrations of unlabelled bFGF. Alternatively, cell glycosaminoglycans were labelled by overnight incubation with 50 mCi/ml [^{35}S]H_2SO_4, the medium was removed and cells were washed with PBS and incubated with unlabelled bFGF for 1 h at 4 °C. The monolayers were washed with PBS and chased for 2 h at 37 °C in medium without bFGF. At the end of this period, the cells were trypsinized, mixed with approx. 1.5×10^7 carrier cells, centrifuged and washed twice with PBS buffer. The cell pellets were resuspended in 50 mM Tris/25 mM KCl/5 mM $MgCl_2$ (pH 7.2) containing 10 mM *N*-ethylmaleimide/1 mM PefaBlock (Boehringer-Mannheim GmbH, Mannheim, Germany)/1 μg/ml leupeptin/0.5 μg/ml pepstatin, and incubated on ice for 15 min [29]. Cells were lysed by incubation with 0.4 % Nonidet P-40/0.4 % deoxycholate and the cytosolic material was separated from nuclei by multiple washing and centrifugation at 1500 ***g*** in 0.25 M sucrose/50 mM Tris/25 mM KCl/5 mM $MgCl_2$ (pH 7.2) [29]. An aliquot of the nuclear pellet was assayed for radioactivity to determine the amount of heparan sulphate or bFGF present in the nucleus.

Controls were performed in order to determine the background caused by non-specific retention of radioactively labelled molecules in the nuclear pellet. For non-specific binding of [^{35}S]heparan sulphate to nuclei, purified glycosaminoglycans were added to the cell lysate before the nuclei were isolated. This background binding, which corresponded to 0.5 % of the added radioactivity, was subtracted from all ^{35}S-labelled samples. For non-specific binding of ^{125}I-bFGF, nuclei were isolated from unlabelled cells that were not exposed to the growth factor, incubated with ^{125}I-bFGF in the presence of carrier for 20 min on ice, and then the nuclei were repurified according to the protocol and the ^{125}I radioactivity associated with the organelle determined. This background binding, which corresponded to 3.3 % of the added radioactivity, was subtracted from all ^{125}I-labelled samples. We also determined whether bFGF treatment enhanced the non-specific binding of ^{35}S-labelled material to the organelle during isolation. Nuclei were isolated from cells that were incubated with 2.5 μg/ml bFGF for 2 h, and then incubated on ice with cytosolic material from ^{35}S-labelled cells that remained in the supernatant after centrifugation of nuclei. After 20 min the isolated nuclei were repurified. After subtraction of non-specific binding, approx. 0.6 % of the added ^{35}S radioactivity purified with the nuclei, suggesting that there is very little non-specific retention due to the presence of bFGF in the organelle.

RESULTS

Degradation and recycling of heparan sulphate chains in the presence and absence of bFGF

Even though they lack the FGF receptor, previous studies have shown that CHO cells are able to internalize bFGF through its interaction with heparan sulphate proteoglycans [10]. This uptake is specific for heparan sulphate proteoglycans, since it can be inhibited if heparin is included in the medium, if glycosaminoglycan sulphation is prevented by adding chlorate to the medium, or if the cell-surface proteoglycans are removed by treatment with heparinase [10]. Since bFGF prevents heparanases from degrading heparan sulphate chains *in vitro* [20], we wanted to determine whether the growth factor would have a similar effect in intact cells. To follow heparan sulphate glycosaminoglycan catabolism, confluent CHO cells were pulse-labelled with [^{35}S]H_2SO_4, incubated for 1 h at 4 °C with various concentrations of bFGF to saturate the binding sites, and chased in non-radioactive media. Heparan sulphate chains were isolated from the cells and medium at various times of chase and purified by anion exchange over a DEAE–Sephacel column. Protein cores were removed by alkaline borohydride treatment, and the [^{35}S]chondroitin sulphate chains were degraded by chondroitin ABC lyase to disaccharides which were then separated from [^{35}S]heparan sulphate glycosaminoglycans by another anion-exchange column. This process also removed any inorganic [^{35}S]sulphate which could be present either as a remnant from the radioactive medium, or as a result of lysosomal catabolism of glycosaminoglycans.

In the absence of growth factor, the amount of heparan sulphate chains associated with the cells decreases rapidly in the first 4 h of chase and then levels off over the next 8 h (Figure 1A). In some experiments glycosaminoglycan catabolism was followed for 20 h (results not shown), and even after that time the amount of cellular [^{35}S]heparan sulphate chains remained at the levels seen at the 12 h chase point. Most of the [^{35}S]heparan sulphate chains lost from the cells are secreted into the medium, rather than being degraded, and the majority of this secretion occurs during the first 4 h of the chase (Figure 1A). Over the course of an average 12 h experiment only 25–30 % of the ^{35}S radioactivity is lost from cells and medium, confirming our previous findings that heparan sulphate degradation in CHO cell lysosomes is slow and inefficient [30]. Since most of the degradation of heparan sulphate occurs in the first 4 h of the chase (Figure 1A), it suggests that CHO cells have a small population of heparan sulphate proteoglycans that are rapidly metabolized, and another population that is degraded more slowly [31]. However, our data do not indicate whether the catabolic rate is determined by the protein core or whether there are other factors, such as interaction with ligands, that may play a role in the turnover.

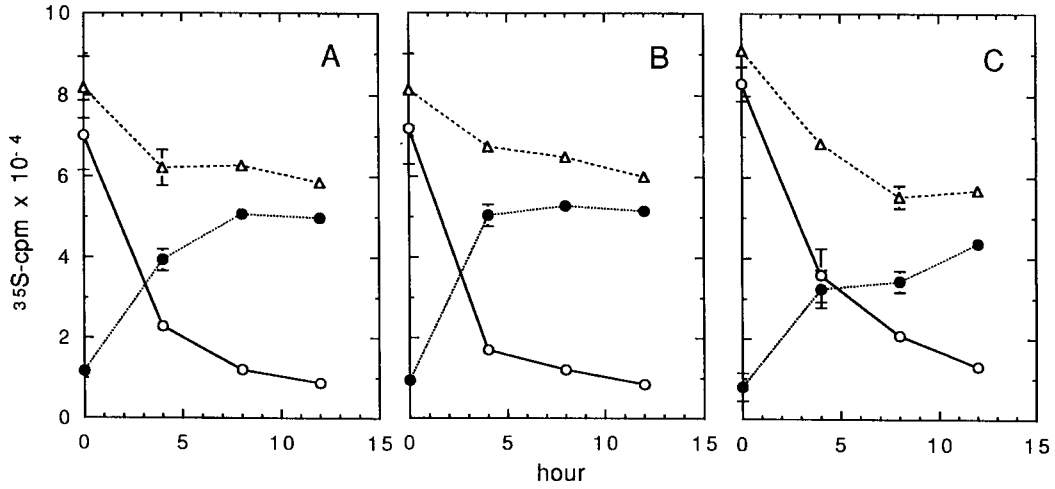

Figure 1 Time course of distribution of heparan sulphate-degradation products in CHO cells in the presence and absence of bFGF

[^{35}S]Heparan sulphate glycosaminoglycans were isolated from CHO cells incubated in the absence of bFGF (**A**), with 5 ng/ml bFGF (**B**) or with 2.5 μg/ml bFGF (**C**). ○, [^{35}S]Heparan sulphate associated with the cells; ●, [^{35}S]heparan sulphate present in the medium; △, [^{35}S]heparan sulphate in the cells and medium. The error bars relate to duplicates within a single experiment. These graphs are representative of three other independent experiments that produced comparable results.

Heparan sulphate proteoglycan catabolism was then examined in the presence of 5 ng/ml (Figure 1B) and 2.5 μg/ml bFGF (Figure 1C). The lower concentration was chosen because it is sufficient for bFGF signalling through tyrosine kinase receptors [32], while the higher concentration approaches the amount of the growth factor required for inhibition of heparan sulphate glycosaminoglycan degradation *in vitro* [20]. At 5 ng/ml bFGF the distribution of cell-associated and medium [^{35}S]heparan sulphate molecules is similar to that of cells cultured without growth factor (Figure 1B), suggesting that at this low concentration, bFGF has little effect on the catabolism of the proteoglycans. The small increase in the relative amount of [^{35}S]glycosaminoglycans secreted from the bFGF cultured cells during the first 4 h of chase is not significant since it is not reproducible. However, when cells are cultured with 2.5 μg/ml bFGF, differences in both the catabolism and the distribution of [^{35}S]heparan sulphate chains were observed (Figure 1C). Instead of seeing the loss of macromolecular ^{35}S radioactivity slow down after the first 4 h of chase, the complete degradation of heparan sulphate chains continues, and does not level off until after 8 h. In addition, more of the [^{35}S]heparan sulphate chains remain associated with cells incubated with 2.5 μg/ml bFGF, resulting in a decrease of the [^{35}S]glycosaminoglycans released into the medium. These results suggest that there may be an interaction between the growth factor and the glycosaminoglycans that prevents the heparan sulphate chains from being exported. Instead, these intracellular chains may be transported to lysosomes, since more of the glycosaminoglycans appear to be degraded.

To establish whether heparan sulphate catabolism by heparanases was affected in the presence of bFGF, heparan sulphate chains isolated from the cells and medium in the presence or absence of the growth factor were purified, and their size was examined by gel-filtration HPLC on a TSK 4000 column (Figures 2 and 3). As previously reported [23], the cell-associated nascent heparan sulphate molecules at the beginning of the chase were intact, eluting as a single peak corresponding to an average molecular mass of 81 kDa (Figure 2A). After 4 h in the absence of bFGF, approx. half of the material associated with the cells were short, 6 kDa chains, whereas the remainder was still present as intact long chains (Figure 2B). With time, the amount of the long glycosaminoglycans gradually decreased, and by 12 h over 90 % of the chains were degraded to short oligosaccharides (Figures 2C and 2D). Unlike the studies *in vitro* [20], the presence of bFGF did not prevent the formation of short chains inside the cells (Figure 2), indicating that the growth factor did not affect the ability of heparanases to cleave the heparan sulphate substrate. As expected from the distribution studies (Figure 1), cells cultured with 2.5 μg/ml bFGF had an increase in cell-associated heparan sulphate chains at all three chase points. By 12 h this increase is due to the accumulation of short heparan sulphate species (Figure 2D).

The heparan sulphate chains isolated from the medium at the beginning of the chase were somewhat shorter than the average nascent glycosaminoglycans (Figure 3A), which are presumably the products formed when heparan sulphate glycosaminoglycans are shed from the cell surface [31,33]. After 4 h there is an increase in these long glycosaminoglycan chains in all three experimental conditions (Figure 3B), suggesting that during this portion of the chase period the primary loss of cell-associated heparan sulphate is due to shedding of cell-surface molecules. Shorter [^{35}S]heparan sulphate species, presumably generated by heparanases, are also observed in the medium of cells cultured without bFGF or in the lower concentration of the growth factor (Figure 3B), and the proportion of these shorter glycosaminoglycans in the secreted population increases at both 8 and 12 h (Figures 3C and 3D). Fewer [^{35}S]heparan sulphate chains are secreted from cells cultured with 2.5 μg/ml bFGF at all time points (Figures 3B–3D), and this is especially apparent with regard to the short, heparanase-derived chains. The shorter [^{35}S]heparan sulphate chains present in the medium may be derived from the action of extracellular heparanases; however, we favour the explanation that they are produced by intracellular enzymes and then recycled to the cell surface and secreted. The amount of long heparan sulphate chains in the medium remains fairly constant throughout the chase period (Figures 3B–3D), suggesting that once secreted or shed from cells the glycosaminoglycans are inert to further catabolism. This idea is supported by

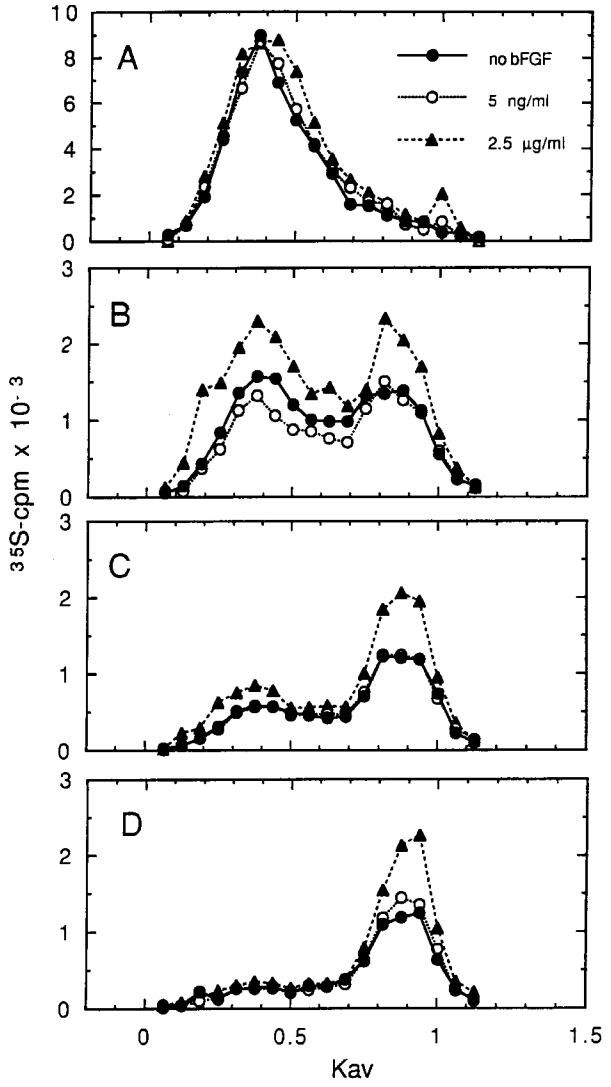

Figure 2 Degradation of cell-associated heparan sulphate glycosaminoglycans in the absence and presence of bFGF

The sizes of ^{35}S-labelled heparan sulphate chains present in the cells at 0 (**A**), 4 (**B**), 8 (**C**) and 12 (**D**) h of the chase were analysed by gel filtration on a TSK 4000 HPLC column. Degradation products generated in the presence of 0 (●), 5 ng/ml (○) and 2.5 μg/ml (▲) bFGF were examined. The graphs are representative of three other independent experiments that produced comparable results.

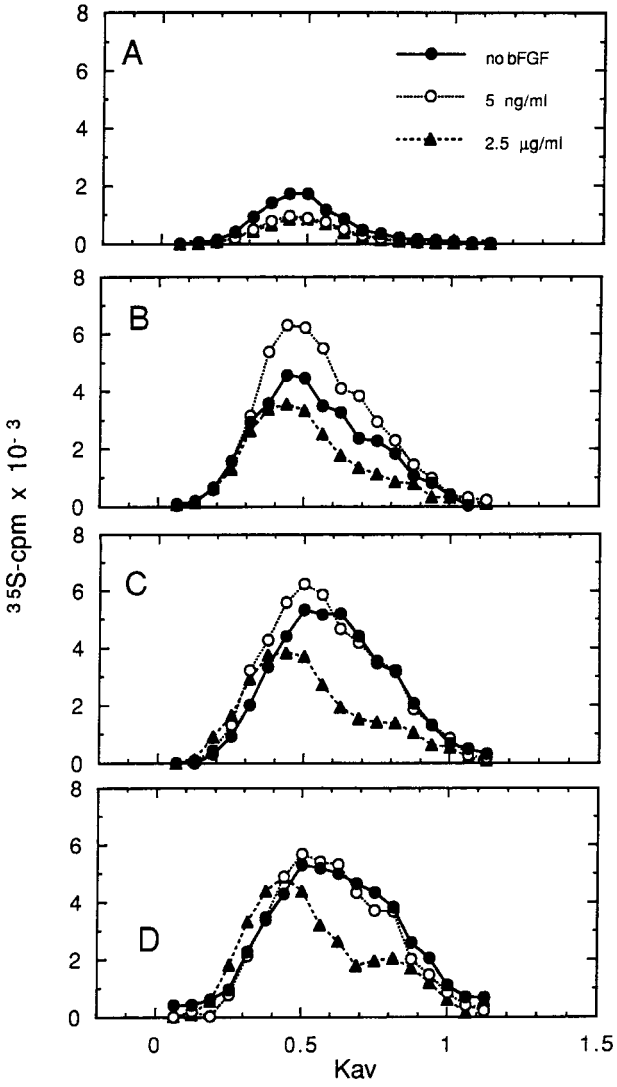

Figure 3 Degradation of heparan sulphate glycosaminoglycans secreted from CHO cells in the absence and presence of bFGF

The sizes of ^{35}S-labelled heparan sulphate chains present in the media at 0 (**A**), 4 (**B**), 8 (**C**) and 12 (**D**) h of the chase were analysed by gel filtration on a TSK 4000 HPLC column. Sizes of glycosaminoglycans secreted in the presence of 0 (●), 5 ng/ml (○) and 2.5 μg/ml (▲) bFGF were examined. The graphs are representative of three other independent experiments that produced comparable results.

experiments that examined whether the size of long, purified heparan sulphate glycosaminoglycans was altered when they were added to cultured CHO cells. Even after 24 h, the size of the [^{35}S]heparan sulphate incubated with cells was comparable to ^{35}S-labelled chains incubated in media alone, indicating that very little degradation of the polysaccharide is occurring once the glycosaminoglycans are released from cells (I. Ventekesan and K. J. Bame, unpublished results). Since the affinity of both long and short heparan sulphate species for bFGF are comparable [20], it is likely that the growth factor remains bound to the glycosaminoglycan inside cells. The reduced secretion of these short heparan sulphate chains and the accumulation of the 6 kDa species inside cells treated with 2.5 μg/ml bFGF is consistent with the hypothesis that the interaction with the growth factor prevented export of the short heparanase-degraded molecules.

Heparan sulphate glycosaminoglycan degradation in endosomes and lysosomes

Since the distribution of heparan sulphate-degradation products between cells and medium suggested that their movement along the endosomal pathway may be altered when they are complexed to bFGF, we decided to characterize in more detail the degradation steps in heparan sulphate proteoglycan catabolism. Indirect evidence shows that the heparanase-catalysed degradation of heparan sulphate chains occurs in endosomes [31,34]. To establish whether this was the intracellular location for the action of CHO heparanases, cells were pulse-labelled with [^{35}S]H_2SO_4, chased in non-radioactive medium, and at various time points the cells were harvested and the organelles isolated by density-gradient centrifugation [25,35,36]. Although the conditions used in these experiments do not separate early and late endosomes,

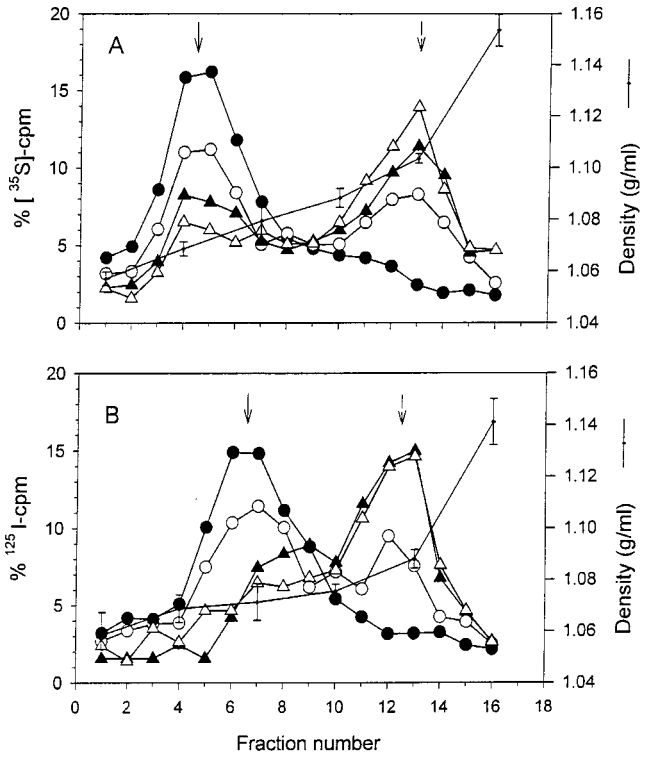

Figure 4 Separation of endosomes and lysosomes from CHO cells by a Percoll density gradient

The gradient fractions were examined for the presence of ^{35}S-labelled molecules (**A**) and ^{125}I-bFGF (**B**). Distribution of labelled molecules was determined at 0 (●), 2 (○), 4 (▲) and 8 (△) h. (**A**) The experiments determining the amount of ^{35}S-labelled material in endosomes and lysosomes were performed in the absence of bFGF; however, the results are comparable with data obtained in the presence of 2.5 μg/ml bFGF. (**B**) CHO cells were incubated with ^{125}I-bFGF in the presence of 5 ng/ml bFGF to follow localization of bFGF. Similar distribution of the ^{125}I-bFGF was obtained when cells were incubated with 2.5 μg/ml bFGF. The arrows indicate peak positions of endosomal (unbroken arrow) and lysosomal (dashed arrow) markers. Fractions 3–7 and 10–15 were pooled as endosomal and lysosomal material, respectively.

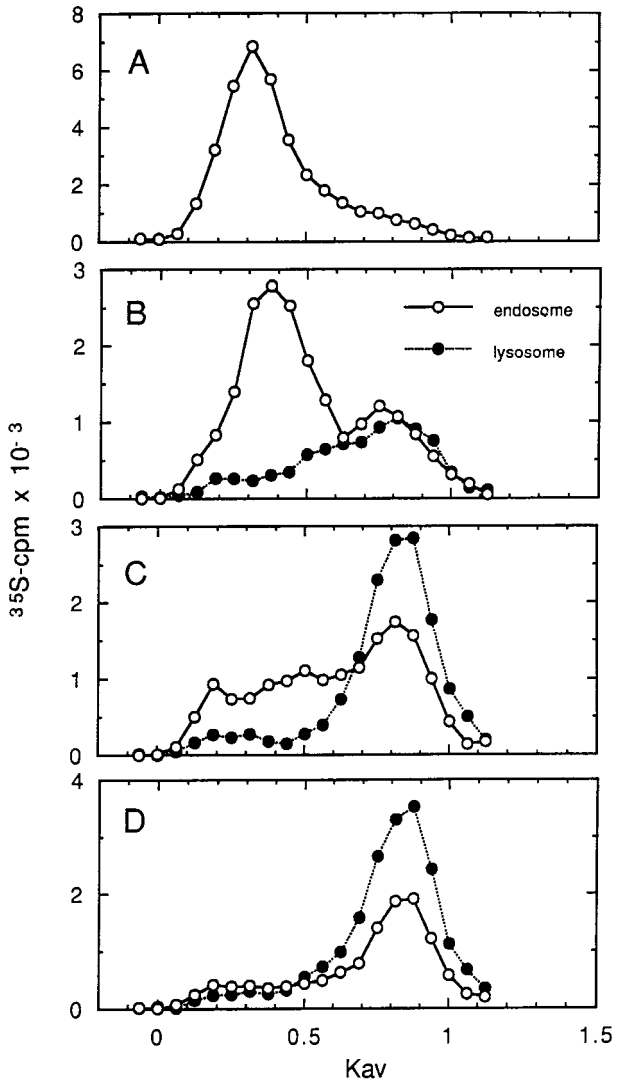

Figure 5 Size of heparan sulphate-degradation products in endosomes and lysosomes

Heparan sulphate chains were isolated from endosomal (○) and lysosomal (●) populations separated by a density gradient (see Figure 4A). The sizes of the purified glycosaminoglycans were analysed by gel filtration on a TSK 4000 HPLC column. (**A**) 0; (**B**) 2; (**C**) 4; and (**D**) 8 h.

they efficiently differentiate between pre-lysosomal and lysosomal organelles. The cellular material was separated into two major fractions by the Percoll gradient (Figure 4). Fractions of lower density (approx. 1.07 g/ml), which co-localized with short-term endocytosed FITC–dextran, should contain primarily endosomal vesicles [36]. Therefore, we refer to them as endosomal fractions, although they most likely contain minor portions of other organelles and membrane fractions [25,37,38]. Material with higher density (1.09–1.10 g/ml) was determined to be of lysosomal origin, using acid phosphatase and β-hexosaminidase activities as markers.

At the beginning of the pulse–chase, ^{35}S-labelled material, which represents both heparan and chondroitin sulphate species, was present exclusively in the endosomal fractions (Figure 4A). These molecules are most likely to be the contaminating cell-surface proteoglycans, since the peak disappeared when the cells were treated with trypsin prior to harvesting (results not shown). After 2, 4 and 8 h, [^{35}S]glycosaminoglycans were present in both endosomes and lysosomes. More radioactive material was present in lysosomes at the later time points (Figure 4A), indicating that the ^{35}S-labelled polysaccharides were being transported through the endosomal pathway. The [^{35}S]heparan sulphate glycosaminoglycans associated with the endosomal and lysosomal fractions were purified away from [^{35}S]chondroitin sulphate chains by treating the purified glycosaminoglycans with chondroitin ABC lyase, as described in the Materials and methods section. Based on the radioactivity associated with the [^{35}S]chondroitin sulphate disaccharides, both heparan sulphate and chondroitin sulphate follow a similar catabolic pathway, indicating that the distribution of the ^{35}S-labelled material in the density gradient (Figure 4A) reflects that of the heparan sulphate species. Initially, all the heparan sulphate species were associated with the endosomal material, but after 2 h, approx. 20% of the cellular heparan sulphate chains moved from endosomes to lysosomes. This amount increased to about 50% after 4 h, and remained close to this value until the end of the 8 h chase.

The size of the [^{35}S]heparan sulphate chains present in the gradient-purified endosomes and lysosomes was examined by TSK 4000 HPLC gel filtration (Figure 5). At the beginning of the chase, the endosome-associated material contained only long, nascent 81 kDa heparan sulphate chains. These glycosamino-

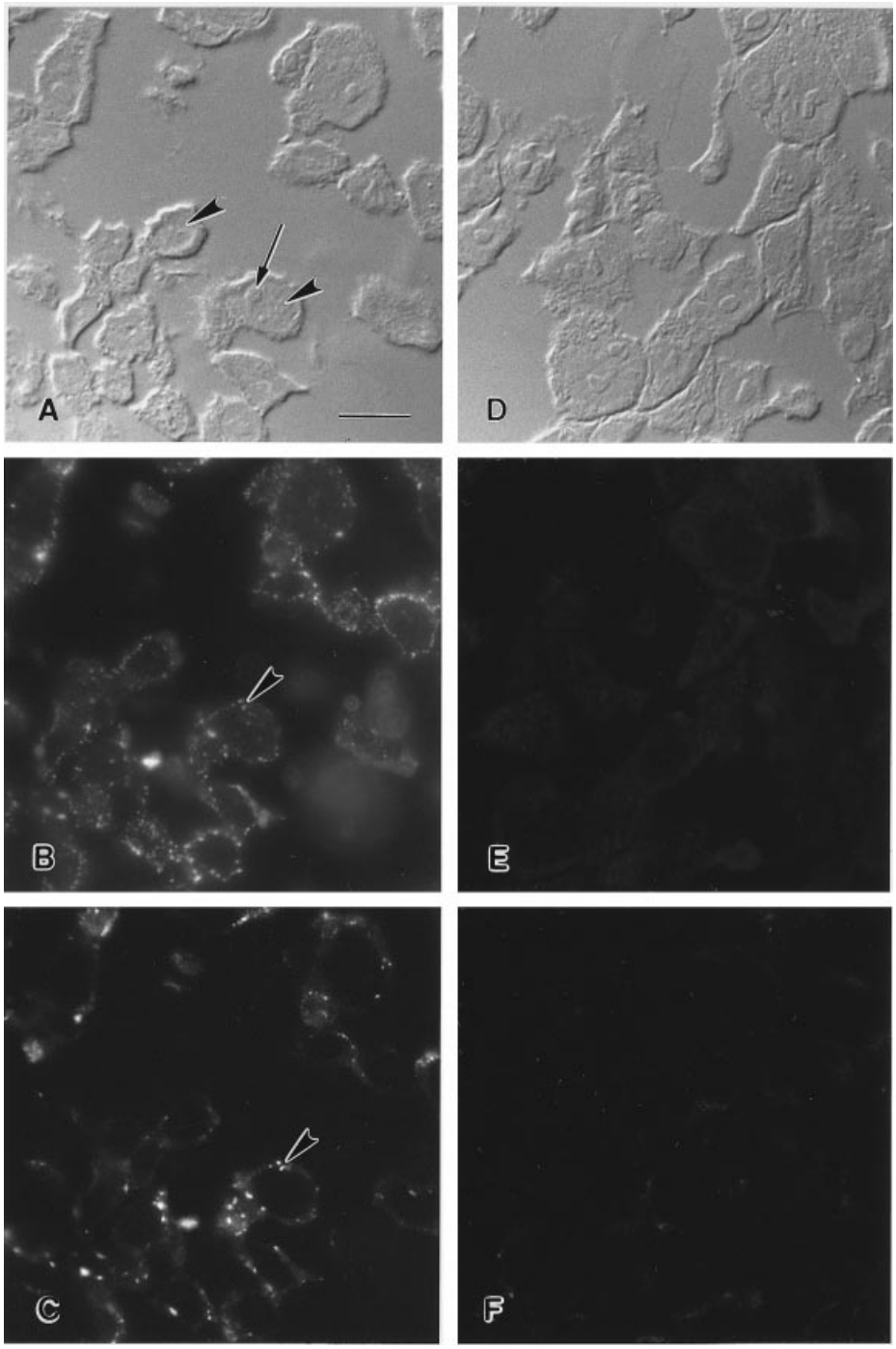

Figure 6 Immunofluorescent labelling of CHO cells

(**A**) Differential interference contrast (DIC) image of field shown in (**B**) and (**C**). Nuclei (arrowheads) and nucleoli (arrow) are distinguishable from cytoplasm; (**B**) anti-heparan sulphate labels broadly distributed perimeter cytoplasmic structures (arrowhead); (**C**) anti-bFGF labels many (arrowhead), but not all of the structures shown in (**B**); (**D**, **E**, **F**) DIC and immunofluorescent images of a similar field, labelled with fluorescent secondary antibodies only. Bar = 10 μM.

glycans were covalently attached to protein cores since they eluted as larger molecules, comparable in size to intact proteoglycans, prior to base treatment [23] (results not shown). After 2 h, a portion of these long, endosomal glycosaminoglycans had been processed to the short species (Figure 5B), and by 8 h nearly all the glycosaminoglycans in the endosomes were short (Figure 5D). Although the proportion of long and short chains in endosomal fractions varied slightly between different experiments (results not shown), in all cases the majority of the long heparan sulphate chains were cleaved to the short 6 kDa species before they arrived at lysosomes. In support of this finding, 75–90 % of the heparan sulphate chains in the gradient-purified lysosomes are the short, 6 kDa species at all chase points (Figure 5). The variations in the endosomal heparan sulphate population, as well

as the lower yield of long heparan sulphate chains than expected from the whole-cell pulse–chase experiments (Figure 2), are likely to be caused by partial removal of cell-membrane material before the Percoll-gradient density centrifugation. The additional steps required for separating and isolating the chains from the intracellular organelles could also contribute to losses of [^{35}S]heparan sulphate species. Therefore, while we cannot conclude that every single heparan sulphate chain is cleaved by heparanases before it reaches lysosomes, our findings support the hypothesis that the primary site for heparanase action is in endosomes [34]. To confirm that heparanases act in the endosomal compartments, fractions from the density gradient were assayed for heparanase activity (results not shown). The activity was found to co-localize with the endosomal peak, bringing additional evidence that the first steps of glycosaminoglycan degradation in CHO cells occur in endosomes.

Distribution of internalized bFGF in endosomes and lysosomes

To follow bFGF internalization, CHO cells were incubated with ^{125}I-bFGF at either 5 ng/ml or 2.5 μg/ml. Since both concentrations of the growth factor gave comparable results, only the findings obtained in the presence of 5 ng/ml bFGF are presented here. Separation of endosomes and lysosomes by density-gradient centrifugation (Figure 4B) indicates that the growth factor follows a similar pathway to the glycosaminoglycans. Initially, bFGF is present only in endosomal fractions (Figure 4B). After 2 h, approx. 30 % of the growth factor is transported to the lysosomes and this amount increases to over 60 % at 8 h (Figure 4B). All of the ^{125}I-labelled material was precipitable by trichloroacetic acid, indicating that the radioactive label was still attached to the protein. A minor shift of the lower-density material, relative to the position of the endosomal markers, was observed at 4 and 8 h of the chase (Figure 4B). This shift could result from the presence of vesicles of different density than endosomes; however, it could also be an artifact caused by density variations in the gradient. These findings show that bFGF is transported through the same endosomal pathway as heparan sulphate glycosaminoglycans, and provide indirect evidence that the two molecules may remain in a complex. The sizes and distribution of the endosomal and lysosomal heparan sulphate-degradation products from cells incubated with 2.5 μg/ml bFGF did not differ significantly from those obtained in the absence of the growth factor (results not shown).

Immunolocalization of bFGF and heparan sulphate chains

Although our pulse–chase studies indicate that the short heparan sulphate glycosaminoglycans and bFGF are transported through similar intracellular compartments, they do not prove that the two molecules are in the same intracellular organelle. To examine the location of heparan sulphate and bFGF inside cells, we double-immunolabelled frozen sections from cells that were incubated with 2.5 μg/ml of the growth factor for 2 h prior to harvesting. Sections were incubated with anti-heparan sulphate and anti-bFGF immunoglobulins and, subsequently, with fluorescently labelled secondary antibodies. The specificity of the primary antibodies was established with cells incubated in the absence of bFGF, and sections treated with heparitinase I, an enzyme that specifically removes heparan sulphate [39,40]. Negligible label was associated with the above control samples (results not shown), indicating that the immunoglobulins were recognizing only the corresponding antigens. Double-labelled sections indicated that heparan sulphate and bFGF were co-localized in the cytoplasm of CHO cells (Figure 6). These regions of co-localization were discrete, or punctate, and located throughout the cytoplasm. No labelling was detected in the nuclei. The pattern of immunolabelling is consistent with the two antigens being found predominantly in the subcellular fractions enriched in endosomal material (Figure 4). In addition to the sites of co-localization with bFGF, anti-heparan sulphate-positive sites appear in a broader, more widely distributed pattern in the cytoplasm. It is unclear if this reflects the absence of bFGF from these sites, or if the growth factor is present, but not detected. The secondary antibodies showed no cross-reactivity (results not shown) and minimal background was observed when primary antibodies were omitted (Figures 6E and 6F).

Table 1 Nuclear localization of bFGF and heparan sulphate

All reported values were obtained as a result of at least two independent experiments, in most cases performed with duplicate concentrations. The values are reported after subtraction of background binding (see Materials and methods). nd, not determined.

	bFGF in the nucleus		^{35}S-Labelled material in the nucleus	
bFGF (ng/ml)	(pg/3 $\times$ 10^5 cells)	(% of intracellular levels)	(c.p.m. $\times$ 10^{-3}/ 3 $\times$ 10^5 cells)	(% of intracellular levels)
None	–	–	21.9 $\pm$ 8.1	2.2 $\pm$ 0.8
4.0–500*	1.6	$\approx$ 2.0	23.3 $\pm$ 4.9	2.3 $\pm$ 0.5
1200	940	3.5	nd	nd
2500	7000–28 000	10–40	80.3 $\pm$ 7.7	8.1 $\pm$ 0.8

* Our detection methods are not sensitive enough to measure significant differences in nuclear ^{125}I-bFGF in this concentration range.

Nuclear targeting of bFGF and heparan sulphate chains

bFGF has been shown to be targeted to the nucleus in some cell lines, including CHO cells [19,21]. Since the pulse–chase experiments indicated that bFGF binding to heparan sulphate chains altered their destination, we examined the possibility that the glycosaminoglycan is transported to the nucleus with the growth factor. First, we examined whether bFGF was transported to the nucleus under our experimental conditions. CHO cells were incubated with ^{125}I-bFGF in the presence of unlabelled growth factor and, after 2 h, the nuclei were isolated and examined for the presence of the radioactively labelled protein (Table 1). At lower concentrations of bFGF, only 2–3 % of the internalized growth factor was detected in the nucleus (Table 1). This amount increased to, on average, 26 % when the cells were incubated with 2.5 μg/ml bFGF, indicating that the growth factor had been transported to the organelle.

Once we confirmed that bFGF was transported to the nucleus, we examined whether heparan sulphate chains accompanied the growth factor to the organelle. Previous experiments in this laboratory established that heparan sulphate chains are not normally found in CHO cell nuclei. In these experiments we compared the amounts of [^{35}S]glycosaminoglycans isolated with nuclei from cells that had been labelled for three days with [^{35}S]H_2SO_4 and unlabelled cells that had purified cell-associated [^{35}S]heparan sulphate [24] added to them before they were lysed (K. J. Bame and N. Midduri, unpublished results). The percentage of [^{35}S]heparan sulphate isolated with the nuclei was very low in both cases (1.4 $\pm$ 1.6 % for the cells incubated with [^{35}S]H_2SO_4 and 1.4 $\pm$ 0.4 % for the added glycosaminoglycan), indicating that under normal conditions CHO cells do not have significant amounts of nuclear heparan sulphate. To determine whether this distribution changes in the presence of bFGF, CHO

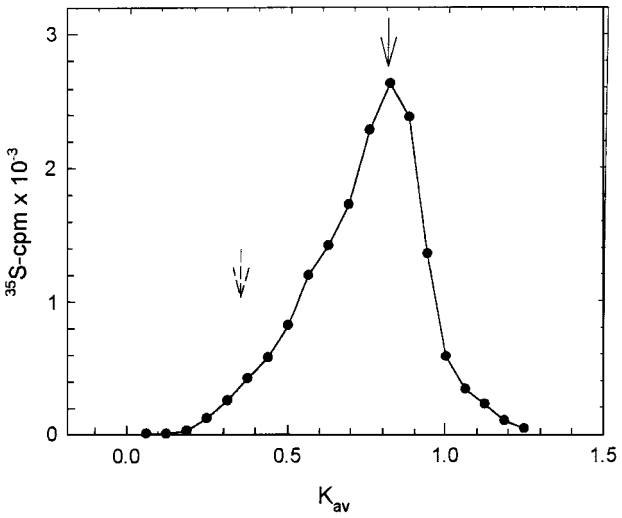

Figure 7 Size of nuclear heparan sulphate chains

[^{35}S]Heparan sulphate glycosaminoglycans targeted to CHO cell nuclei in the presence of 2.5 μg/ml bFGF were purified, and their size examined by gel filtration on the TSK 4000 HPLC column. The arrows indicate the elution position of short 6 kDa (unbroken arrow) and nascent (dashed arrow) glycosaminoglycans from CHO cells.

cells were incubated overnight with [^{35}S]H_2SO_4 to label glycosaminoglycans, exposed to various concentrations of the growth factor for 2 h, then the nuclei were purified and examined for the presence of sulphated glycosaminoglycan species. As with the previous studies, only low amounts of ^{35}S-labelled material were present in the nuclei isolated from cells cultured in the absence of the growth factor (Table 1). The amount of nucleus-associated ^{35}S-labelled material increased only slightly at low bFGF concentrations, and is not statistically significant. However, in the presence of 2.5 μg/ml bFGF, 8.1 % of the cell-associated ^{35}S-labelled material was localized in this organelle (Table 1). This value may actually be closer to 7.5 %, since control experiments indicate that 0.6 % of ^{35}S-labelled material present in the nuclear pellet may be retained due to non-specific binding of ^{35}S-glycosaminoglycans to bFGF-treated nuclei (see Materials and methods). The ^{35}S-labelled material associated with the nuclei was purified, and was shown to consist mainly of short, 6 kDa heparan sulphate chains (Figure 7), suggesting that the complex between the growth factor and heparanase-degraded chains was translocated to the nucleus. To demonstrate an interaction between heparan sulphate chains and bFGF, we attempted to cross-link the two molecules inside the nuclei. However, incubation of purified radioactively labelled nuclear material with cross-linking reagents resulted in the formation of high-molecular-mass complexes that could not be resolved on polyacrylamide gels and bound irreversibly to anion-exchange columns (results not shown). These aggregates could be multimeric complexes between the growth factor and heparan sulphate and/or other molecules, since it is likely that once inside the nucleus bFGF interacts with a variety of nuclear components.

Interestingly, the immunofluorescence experiments did not detect either heparan sulphate or bFGF in the nucleus of CHO cells (Figure 6), even though the experimental conditions used in those studies were similar to the nucleus-purification experiments. It may be that the amounts of the heparan sulphate and bFGF antigens in the nucleus are not high enough to be detected by the antibodies, or were masked by other proteins and nucleic acids in the organelle.

DISCUSSION

Heparan sulphate proteoglycans have been shown to bind a variety of ligands [3–9] and it is conceivable that these interactions might affect the catabolism of the glycosaminoglycans by intracellular heparanases. We examined this possibility using bFGF as our model ligand, since it was shown to be internalized by binding cell-surface heparan sulphate proteoglycans [10,16], and because it prevented heparanases from cleaving heparan sulphate chains *in vitro* [20]. Our studies show that bFGF is internalized (Figure 4) and co-localized with heparan sulphate glycosaminoglycans in CHO cells (Figure 6), but it does not inhibit heparan sulphate degradation (Figure 2). Multiple growth-factor-binding sites need to be occupied in order to inhibit heparan sulphate glycosaminoglycan catabolism *in vitro* [20]. Since bFGF binding to heparan sulphate is reversible [32,41], it is possible that much higher concentrations of the growth factor would be required to saturate the binding sites on the glycosaminoglycan chains in endosomes. Under these conditions heparanases may be able to cleave the glycosaminoglycan when the bFGF molecules dissociate from the heparan sulphate. Additional factors could also be present inside the cells that might reverse bFGF's inhibitory effects.

Although the internalized bFGF does not prevent heparanases from cleaving the glycosaminoglycan, our studies show that high concentrations of the growth factor alter the cellular destination of the degraded heparan sulphate chains. In CHO cells, the majority of the heparanase-degraded heparan sulphate species are secreted into the media (Figures 1–3), where they appear to be metabolically inert. The remaining short glycosaminoglycans are localized to vesicles that have a density similar to lysosomes (Figure 4), and remain as macromolecular species for 48–72 h [30]. Under normal conditions there is no evidence that heparan sulphate glycosaminoglycans are present in the nucleus of CHO cells (K. J. Bame and N. Midduri, unpublished results). When cells are cultured with 2.5 μg/ml bFGF, heparan sulphate glycosaminoglycans are still cleaved to short chains, but they are not secreted to the same extent as cells cultured without the growth factor (Figure 1), indicating that the interaction with bFGF has prevented their export. Additionally, 8.1 % of the degraded ^{35}S-labelled chains are now found in purified nuclei, presumably transported to the organelle along with the growth factor. Unlike higher-molecular-mass forms of the growth factor, the 18 kDa bFGF used in this and other studies [19,21] does not contain any known nuclear localization signal. It is not clear what mechanism delivers this form of bFGF to the nucleus; however, our study indicates that during transport the growth factor probably remains bound to heparan sulphate chains. This interaction may be necessary to protect bFGF from inactivation [17,18].

Based on these results, we propose a model for distribution of internalized bFGF and heparan sulphate-degradation products in CHO cells (Figure 8). When bFGF-bound cell-surface proteoglycans are internalized, the growth factor remains associated with the glycosaminoglycan chains. In endosomes, heparanases cleave the glycosaminoglycan chains from the protein core and degrade them to 6 kDa pieces. In contrast to the studies *in vitro* [20], bFGF does not interfere with this degradation process inside the cells. However, since all the short heparan sulphate chains were previously shown to bind bFGF with similar affinity to the nascent chains [20], it is likely that the growth factor remains bound to the short glycosaminoglycan species. Because

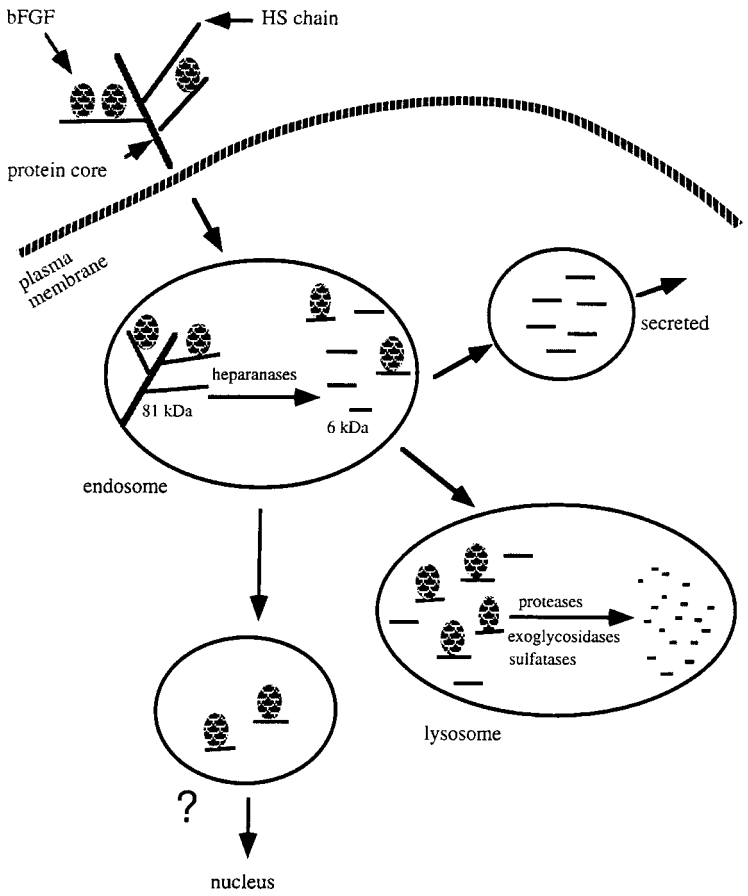

Figure 8 Model for distribution of internalized bFGF and heparan sulphate degradation products

Cell-surface heparan sulphate (HS) proteoglycans, with or without bound bFGF, are internalized into the endosomal pathway. In endosomes the glycosaminoglycans are removed from the core protein and cleaved to short chains by heparanases. Some of these short heparan sulphate chains will have bFGF bound to them [20], and the destination of the glycosaminoglycan will depend on whether it is associated with the growth factor. Both free and bFGF-bound heparan sulphate will be transported to lysosomes where they will be degraded to monosaccharides and free sulphate. The bound growth factor inhibits the secretion of the short heparan sulphate chains, but allows a fraction of the glycosaminoglycans to be delivered to the nucleus (see text for details).

high concentrations of bFGF are required to saturate all the binding sites on the glycosaminoglycan chains, there should be a mixture of free and bFGF-bound heparan sulphate-degradation products inside the cells. These glycosaminoglycans are sorted in endosomes to be either degraded in lysosomes or secreted out of the cell. Only free glycosaminoglycan chains can be secreted. Most of the free and bFGF-bound heparan sulphate species are directed to lysosomes and degraded; however, a fraction of bFGF–heparan sulphate complex is delivered to the nucleus (Figure 8). This specific targeting of heparan sulphate is a result of the interaction with bFGF, since normally CHO cells do not have nuclear transport of heparan sulphate chains.

Because of the high concentrations of bFGF required to observe changes in glycosaminoglycan recycling and the detection of significant amounts of heparan sulphate and bFGF in the nucleus, it is not likely that similar quantitative effects would occur *in vivo*. However, this work demonstrates that the interaction with a ligand, such as bFGF, can affect sorting of heparan sulphate chains during the degradation/recycling pathway. In this case, the complex of the heparan sulphate glycosaminoglycan and its ligand is prevented from being exported from the cell, which may be relevant to the hypothesis that the primary purpose of heparan sulphate proteoglycan-mediated uptake of bFGF is to rapidly clear the growth factor from the extracellular space. However, since the complex can be translocated to the nucleus, where bFGF is proposed to act in an alternative signal transduction pathway, it is possible that the formation of bFGF–heparan sulphate complexes fulfils additional functions. If, indeed, bFGF is acting as a transcription factor [21], then very low amounts of the growth factor in the nucleus would be required to observe biological effects. The association of bFGF with heparan sulphate may be essential for delivery of the growth factor to this organelle.

Since bFGF is only one of several ligands that are internalized while bound to heparan sulphate proteoglycans, these observed effects may have more general applications. It is well established that the activity and stability of glycoproteins is affected by the glycosidic portion of the molecule. For some heparan sulphate ligands, the glycosaminoglycan appears to have a similar role, except it is not covalently attached to the protein ligand. This association may not only protect the biological activity of the ligand, but also facilitate interactions of the ligand with other proteins [9,41]. Since the heparan sulphate chain can exist either as a free glycosaminoglycan or as a component of a proteoglycan, both of these forms can bind ligands with different results. Proteoglycans anchor ligands at the cell surfaces or in the extracellular matrix and mediate internalization and degradation of molecules, such as lipoprotein lipase [8,42], thrombospondin

[7] and hepatic lipase [9], whereas the interaction of protein with short heparan sulphate species may enable its transport to various destinations, during which its activity is protected [4,43]. Short glycosaminoglycan species can be produced by heparanases acting on cell-surface or matrix proteoglycans, or generated by intracellular catabolism and secreted from the cell. Alternatively, the activity-preserving complex between ligand and heparan sulphate glycosaminoglycan could be a direct result of heparanases cleaving the ligand-bound proteoglycans in the matrix [44], on the cell-surface [4], or inside the cells, as shown in this work. The importance of heparan sulphate catabolism for its ligand's biological activity was recently demonstrated in studies examining the interaction between heparan sulphate and lipoprotein lipase in endothelial cells [8]. It was shown that an endothelial cell heparanase can liberate sub-endothelial heparan sulphate-bound lipoprotein lipase, and that specific heparan sulphate oligosaccharides produced by this enzyme facilitate the intercellular movement of the ligand [4]. Although this process occurs at the cell surface, similar mechanisms may be involved in recycling internalized lipoprotein lipase.

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REFERENCES

1 Wight, T. N., Kinsella, M. G. and Qwarnstrom, E. E. (1992) Curr. Opin. Cell Biol. **4**, 793–801
2 Couchman, J. R. and Woods, A. (1996) J. Cell. Biochem. **61**, 578–584
3 Maccarana, M., Casu, B. and Lindahl, U. (1993) J. Biol. Chem. **263**, 23898–23905
4 Pillarisetti, S., Paka, L., Sasaki, A., Vanni-Reyes, T., Yin, B., Parthasarathy, N., Wagner, W. D. and Goldberg, I. J. (1997) J. Biol. Chem. **272**, 15753–15759
5 Parthasarathy, N., Goldberg, I. J., Sivaram, P., Mulloy, B., Flory, D. M. and Wagner, W. D. (1994) J. Biol. Chem. **269**, 22391–22396
6 Lortat-Jacob, H., Turnbull, J. E. and Grimaud, J. A. (1995) Biochem. J. **310**, 497–505
7 Murphy-Ullrich, J. E., Westrick, L. G., Esko, J. D. and Mosher, D. F. (1988) J. Biol. Chem. **263**, 6400–6406
8 Saxena, U., Klein, M. G. and Goldberg, I. J. (1990) J. Biol. Chem. **265**, 12880–12886
9 Kounnas, M. Z., Chappell, D. A., Wong, H., Argraves, W. S. and Strickland, D. K. (1995) J. Biol. Chem. **270**, 9307–9312
10 Roghani, M. and Moscatelli, D. (1992) J. Biol. Chem. **267**, 22156–22162
11 Basilico, C. and Moscatelli, D. (1992) Adv. Cancer Res. **59**, 115–165
12 Johnson, D. E. and Williams, L. T. (1993) Adv. Cancer Res. **60**, 1–41
13 Yayon, A., Klagsbrun, M., Esko, J., Leder, P. and Ornitz, D. M. (1991) Cell **64**, 841–848
14 Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992) Mol. Cell. Biol. **12**, 240–247
15 Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991) Science **252**, 1705–1708
16 Reiland, J. and Rapraeger, A. C. (1993) J. Cell Sci. **105**, 1085–1093
17 Gospodarowitz, D. and Cheng, J. (1986) J. Cell. Physiol. **128**, 475–484
18 Sommer, A. and Rifkin, D. B. (1989) J. Cell. Physiol. **138**, 215–220
19 Baldin, V., Roman, A.-M., Bosc-Bierne, I., Amalric, F. and Bouche, G. (1990) EMBO J. **9**, 1511–1517
20 Tumova, S. and Bame, K. J. (1997) J. Biol. Chem. **272**, 9078–9085
21 Amalrich, F., Bouche, G., Bonnet, H., Brethenou, P., Roman, A. M., Truchet, I. and Quarto, N. (1994) Biochem. Pharmacol. **47**, 111–115
22 Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V. and Basilico, C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4378–4382
23 Bame, K. J. (1993) J. Biol. Chem. **268**, 19956–19964
24 Bame, K. J. and Esko, J. D. (1989) J. Biol. Chem. **264**, 8059–8065
25 Galloway, C. J., Dean, G., Fuchs, R. and Mellman, I. (1988) Methods Enzymol. **157**, 601–611
26 Vaes, G. (1966) Methods Enzymol. **8**, 509–514
27 Hall, C. W., Liebaers, I., Di Natale, P. and Neufeld, E. F. (1978) Methods Enzymol. **50**, 439–455
28 Slot, J. W., Geuze, H. J. and Weerkamp, A. J. (1988) Methods Microbiol. **20**, 211–236
29 Beck, L. A., Hosick, J. J. and Sinensky, M. (1988) J. Cell Biol. **107**, 1307–1316
30 Bame, K. J. and Robson, K. (1997) J. Biol. Chem. **272**, 2245–2251
31 Yanagishita, M. and Hascall, V. C. (1984) J. Biol. Chem. **259**, 10270–10283
32 Moscatelli, D. (1992) J. Biol. Chem. **267**, 25803–25809
33 Ihrcke, N. S. and Platt, J. L. (1996) J. Cell. Physiol. **168**, 625–637
34 Brauker, J. H. and Wang, J. L. (1987) J. Biol. Chem. **262**, 13093–13101
35 Galloway, C. J., Dean, G. E., Marsh, M., Rudnick, G. and Mellman, I. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3334–3338
36 Roff, C. F., Fuchs, R., Mellman, I. and Robbins, A. R. (1986) J. Cell Biol. **103**, 2283–2297
37 Merion, M. and Sly, W. S. (1983) J. Cell Biol. **96**, 644–650
38 Merion, M., Schlesinger, P., Brooks, R. M., Moehring, J. M., Moehring, T. J. and Sly, W. S. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5315–5319
39 David, G., Bai, X. M., Van Der Schueren, B., Cassiman, J.-J. and Van Den Berghe, H. (1992) J. Cell Biol. **119**, 961–975
40 Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D. and Gallagher, J. T. (1990) Biochemistry **29**, 2611–2617
41 Nugent, M. A. and Edelman, E. R. (1992) Biochemistry **31**, 8876–8883
42 Berryman, D. E. and Bensadoun, A. (1995) J. Biol. Chem. **270**, 24525–24531
43 Ruoslahti, E. and Yamaguchi, Y. (1991) Cell **64**, 867–869
44 Vlodavsky, I., Bar-Shavit., R., Ishai-Michaeli, R., Bashkin, P. and Fuks, Z. (1991) Trends Biochem. Sci. **16**, 268–271

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