Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development

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SUMMARY

Kidney epithelia have separate origins; collecting ducts develop by ureteric bud growth and arborisation, nephrons by induced mesenchyme-epithelium transition. Both express sulphated glycosaminoglycans (GAGs) which are strikingly upregulated during nephron differentiation. However, sodium chlorate, an inhibitor of GAG sulphation, and the GAG-degrading enzymes heparitinase plus chondroitinase, did not prevent nephron development. In contrast, ureteric bud growth and branching were reversibly inhibited by the above reagents, the inhibition correlating quantitatively with sulphated GAG deprivation caused by a range of chlorate concentrations. Growth and branching could be independently restored during GAG deprivation by hepatocyte growth factor and phorbol-12-myristate acetate (PMA) respectively. Together these signalling effectors stimulated both branch initiation and growth. Thus growth and morphogenesis of ureteric bud involve distinct signalling pathways both regulated by GAGs.

Key words: kidney development, morphogenesis, branching, glycosaminoglycans, PKC, hepatocyte growth factor, mouse

INTRODUCTION

Little is known about the mechanisms that regulate epithelial differentiation and morphogenesis during embryonic development (for reviews see Fleming, 1992). Of the systems available to study these phenomena, embryonic kidneys offer particular advantages because they contain two epithelia with distinct origins: urinary collecting ducts form by growth and arborisation of an epithelial ureteric bud, while excretory nephrons differentiate via a mesenchyme-epithelium transition induced by the invading ureteric bud (Grobstein, 1953). The latter process is characterised by precisely timed changes in gene expression resulting in sequential synthesis of a number of potentially important molecules such as transcription factors, signal receptors, adhesion molecules and extracellular matrix proteins (Davies and Garrod, 1995). Specific inhibition of these molecules should reveal the developmental function of each, and contribute much to our understanding of how an epithelium is made.

Of all potential morphoregulatory molecules identified in developing nephrons, the proteoglycan, syndecan-1, shows the earliest increase in expression. Syndecan-1 upregulation correlates with the first morphological event of nephron formation, mesenchymal condensation, and precedes expression of epithelial characteristics (Vainio et al., 1989, 1992; Davies and Garrod, 1995). The timing and magnitude of syndecan-1 elevation suggest that the proteoglycan might play a critical role in the developmental process; possible functions include cell-cell adhesion by homotypic binding (Fransson et al., 1982), cell-matrix adhesion via collagen I/collagen III/fibronectin/tenascin binding (Koda et al., 1985; Saunders and Bernfield, 1988; Salmivirta et al., 1991), or presentation of growth factors to high affinity receptors (Rapraeger et al., 1991; Lyon et al., 1994; Gallagher, 1994). Inhibition of proteoglycan function would be expected to block nephrogenesis at or about the time of condensation, and thus indicate its role in normal development.

Some proteoglycan functions are centred on the core protein and others on the glycosaminoglycan (GAG) chains (for reviews see Gallagher et al., 1986; Gallagher, 1989; Kjellén and Lindall, 1991). Techniques that can interfere with just one structural aspect of these complex molecules allow a finer examination of their functional roles. We have used the specific sulphation inhibitor, sodium chlorate (Farley et al., 1978; Rapraeger et al., 1991) and the enzymes heparitinase and chondroitinase ABC, to inhibit the expression of sulphated glycosaminoglycan side chains in mouse kidney rudiments developing in culture. Surprisingly, we find that induction and maturation of nephrons occur in the presence of these inhibitors, whereas both growth and morphogenesis of the ureteric bud are dependent on sulphated GAG synthesis, the two processes being to some extent separable. Our findings identify critical roles for these large polysaccharides in the kidney and highlight similarities and differences between this organ and others that contain branching epithelia.

MATERIALS AND METHODS

Organ culture

The method of organ culture was based on that of Saxén et al. (1965). Metanephric kidney rudiments were isolated from 11-day old embryos of MF1 mice, and were cultured for 8-120 hours on 1 μm nuclepore filters (Costar) placed on Trowell screens at the surface of culture medium as described by Davies (1994). For various experimental treatments, the medium (Eagle's MEM with Earle's salts, non-essential amino acids and 10% foetal calf serum) was supplemented with one or more of the following; 0-40 mM sodium chlorate (AnaLaR, BDH); 0.33-0.67 U/ml heparitinase (Heparinase III; E.C. 4.2.2.8, Sigma cat# H8891); 0.33-0.67 U/ml chondroitinase ABC (E.C. 4.2.2.4, Sigma cat# C2905); 0.5-5 mM dibutyryl cyclic AMP (Sigma cat# D0627); 5-50 ng/ml staurosporine (Sigma cat# S4400); 1-1000 ng/ml phorbol 12-myristate acetate (PMA, Sigma cat# P8139); 2-10 mM sodium sulphate (AnaLaR, BDH), 500 nM methotrexate (Sigma cat# A6770), 2-150 ng/ml bFGF (Sigma cat# F7395), 200-2000 ng/ml EGF (Sigma cat# E4127), 0.1-10 ng/ml TGFβ1 (Sigma cat# T1654), 30-100 ng/ml human HGF (Collaborative Biomedical cat# 40100).

Immunohistochemistry

Cultures were fixed in −20°C methanol, washed in PBS, and stained as whole mounts, rather than being sectioned, so that the anatomy of the entire organ could be examined, and plane-of-section effects could not alter the shapes and apparent dimensions of tubular structures. Fixed nephric rudiments were incubated in primary antibody for 3 hours at 37°C. Antibody dilutions (in PBS) were; syndecan-1 (mAb 281-2) 1/5, laminin 1/1000; CD15 (mAb C3D-1; Sigma cat# C7798) 1/100; calbindin-D-28K (mAb CL-300; Sigma cat# C8666) 1/100; heparan sulphate (mAb 10E4; David et al., 1992) 1/300; heparitinase 'stubs' (mAb 3G10; David et al., 1992) 1/100; chondroitin sulphate (mAb CS-56, specific to chondroitin not dermatan sulphate chains; Sigma cat# C8035) 1/100; α6-integrin (mAb GoH3) 1/10; E-cadherin (mAb DECMA-1; Sigma cat# U2354) 1/800; murine c-met (Santa Cruz, USA) 1/30. Specimens were washed in PBS, incubated in 1/100-1/200 FITC-conjugated secondary antibody for 2 hours at 37°C, washed in PBS, mounted in glycerol and viewed under a Zeiss Axioplan fluorescence microscope or Biorad MRC600 confocal microscope. Controls, which received only secondary antibody or irrelevant primary antibody, showed no fluorescence.

Biochemical assay of glycosaminoglycan synthesis

Cultures of whole kidney rudiments were set up as above, in unlabelled medium containing 0, 10, 20 or 30 mM chlorate (5 kidneys in each). After 4 hours, [^{3}H]glucosamine was added to 10 μCi/ml and at 40 hours, developing kidney rudiments were removed, washed 3× in 3 mls PBS, transferred to 50 μl 1% Triton X-100 in PBS and incubated for 1 hour at 4°C. Samples were frozen, thawed, and treated with 1 mg/ml proteinase K at 37°C overnight. After dilution to 500 μl with PBS the digests were centrifuged at 14000 r.p.m. for 10 minutes. The GAG-containing supernatant was recovered from the DNA pellet and loaded onto an FPLC-linked Mono-Q anion-exchange column (Pharmacia; 1 ml volume) run at a flow rate of 1 ml/minute. After a 5-ml wash with 0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.2, the column was eluted with a gradient (30 ml volume) of 0.15-1.5 M NaCl in 10 mM sodium phosphate, pH 7.2. Fractions of 0.5 ml were collected and analysed by liquid scintillation counting.

BrdU incorporation

Cultures of kidney rudiments were set up as above, in standard medium and medium supplemented with 30 mM sodium chlorate. After 20 hours of incubation, BrdU was added to a final concentration of 100 μM, and 4 hours later the cultures were removed, washed briefly in standard medium and fixed overnight in 4% paraformaldehyde. They were then washed in PBS, incubated in 0.5 mg/ml trypsin (E.C. 3.4.21.4) for 20 minutes at 37°C, refixed in 4% formalin for 20 minutes at room temperature and washed in PBS. To denature their DNA, samples were incubated in 95% formamide, 5% 0.15 M trisodium citrate for 1 hour at 70°C, washed in PBS, and incubated overnight in PBS, containing 1/1000 anti-laminin (Sigma cat# L9393) and 1/20 anti-BrdU (mAb BU-33, Sigma cat# B2531). Cultures were then stained with secondary antibodies as above, and viewed on a Biorad MRC600 confocal microscope.

In situ hybridisation

Whole-mount in situ hybridisation was performed using a method based on that of Wilkinson (1992). Cultures of kidney rudiments were fixed in 4% paraformaldehyde in PBS at 4°C overnight, then washed in PBS 0.1% Tween 20 (PBT), transferred to ×20°C methanol for 5 minutes and washed again in PBT. They were then incubated for 1 hour in 6% H$_2$O$_2$ in PBS, washed 2× 5 minutes in PBT, incubated in 10 μg/ml proteinase K for 15 minutes at 37°C, refixed in 4% formalin and washed in PBT. They were then probed with sense and antisense riboprobes for c-ros and c-ret using an 'RNA Colour Kit' (Amersham International), which uses fluoresceinated probes and an alkaline-phosphatase-conjugated antibody, according to the maufacturer's instructions; sense probes yielded no signal.

RESULTS

Chlorate inhibits ureteric bud branching but not nephron development

In standard medium, embryonic kidney rudiments followed a developmental programme similar to that seen in vivo; the ureteric bud grew and branched successively to generate a tree-like structure, and induced nephrogenic mesenchyme cells to differentiate into epithelial nephrons (Fig. 1A; for recent reviews of kidney development see Davies, 1993; Bard et al., 1994). Heparan and chondroitin sulphates were expressed strongly in all epithelial basement membranes and more weakly throughout the mesenchymal matrix (Fig. 1B,C). Syndecan-1 core protein showed a more restricted distribution, being expressed strongly by ureteric bud tips and the mesenchymal cells surrounding them, but more weakly elsewhere (Fig. 1D).

Synthesis of sulphated glycosaminoglycans can be inhibited with chlorate, which competes with sulphate in the synthesis of phosphoadenosine 5′-phosphosulphate (PAPS), the sulphate donor utilised by the sulphotransferases that mediate polysaccharide sulphation (Farley et al., 1978; Rapraeger et al., 1991). Kidneys developing in culture were treated with a range of chlorate concentrations from 0-50 mM. Concentrations above 40mM caused widespread disruption and death of cells, probably due to simple toxicity rather than apoptosis; propidium iodide staining of cellular remains revealed that most nuclei did not show the condensation typical of programmed cell death (not shown). Development of those treated with 15-30 mM chlorate was reversibly blocked in a very specific manner. Ureteric buds neither branched nor showed obvious growth although their ends frequently produced long, thin, laminin-rich filaments (Fig. 1E). In contrast, the induction and differentiation of nephrons continued apparently normally, although they were reduced in number. Thus the growth-arrested ureteric bud was surrounded by a corona of tubules which showed characteristic curving morphogenesis, and

matured at least to the stage of proximal tubule differentiation (indicated by CD15 expression: Bard and Ross, 1991) (Fig. 1F). In some cultures, certain nephrons developed close to the ureteric bud, and eventually connected with it, as they would in vivo (Figs 1F, 6E). The lower number of nephrons in chlorate-treated cultures is probably due to the reduced area of
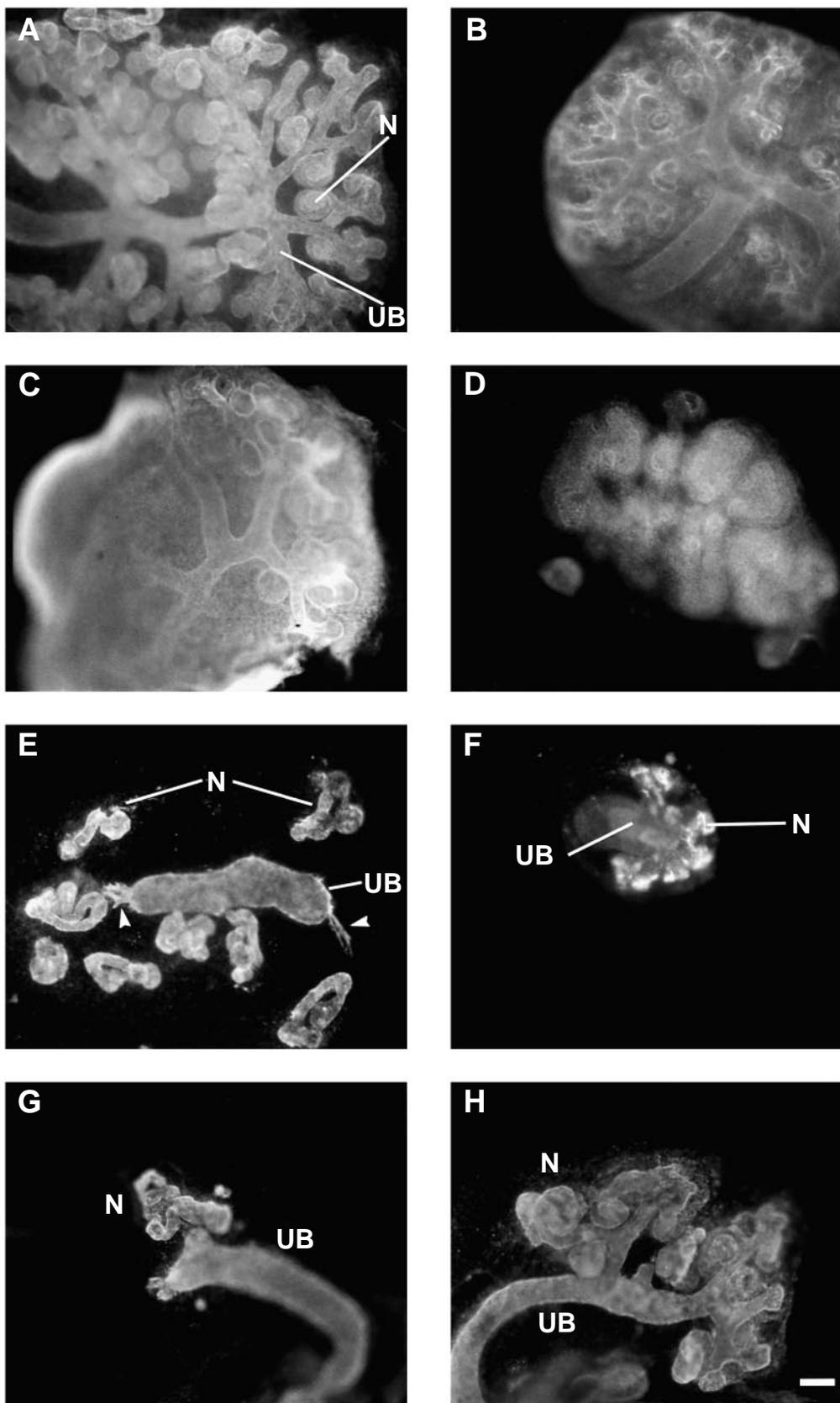
Fig. 1. (A) Embryonic kidney cultured in standard medium for 96 hours, and stained with anti-laminin. The ureteric bud (UB) has branched and induced the differentiation of nephrons (N). (B) Expression of heparan sulphate in kidney rudiments cultured in standard medium; anti-heparan sulphate stains basement membranes of all epithelia strongly, and the mesenchymal matrix more weakly. (C) Expression of chondroitin sulphate chains, which is similar to that of heparan sulphate (Fig. 1b). (D) Expression of syndecan-1 core protein, which is strong in the ureteric bud tips and the mesenchyme condensing around them, but much weaker elsewhere. The pattern is very similar to that described by Vainio et al. (1989). (E) Embryonic kidney cultured in 30 mM chlorate for 96 hours, and stained with anti-laminin. Nephrons (N) have formed normally, but the ureteric bud (UB) has neither grown nor branched, although its ends have produced a number of long fine processes (arrowheads). (F) Expression of proximal tubule marker CD15 by nephrons (N) of kidney rudiments cultured in 30 mM chlorate for 140 hours. Because the sample is thicker than others shown in these micrographs, it had to be photographed at a magnification 40% lower. (G,H) Embryonic kidneys cultured in 30 mM chlorate for 96 hours (G), or for 30 hours followed by 66 hours in standard medium (H), and stained for laminin; ureteric bud (UB) growth and branching was prevented in the continuous presence of chlorate, but took place in the samples from which chlorate had been removed. Scale bar, 100 µm.
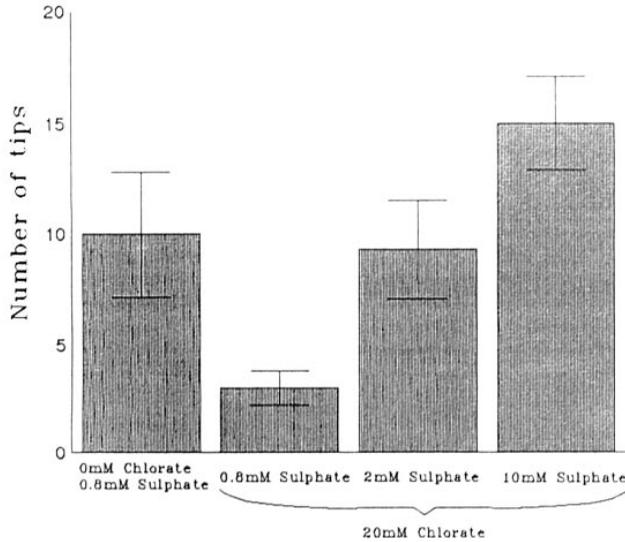
Fig. 2. Competition between chlorate and sulphate ions. The graph shows the effect of different chlorate/sulphate mixtures on ureteric bud branching, quantified by counting the number of branch tips formed; addition of sulphate ions to the growth medium makes chlorate a much less effective inhibitor of ureteric bud development, as expected if chlorate is inhibiting synthesis of the sulphate donor used by sulphotransferases. Standard medium has 0.8 mM sulphate.

ureteric bud able to induce nephrogenesis, induction being mediated by cell-cell contact (Lehtonen, 1976; Saxén and Lehtonen, 1978).

The inhibition of ureteric bud development by chlorate ions was reversible on transfer of chlorate-treated kidney rudiments to standard medium (Fig. 1G,H). Chlorate is a relatively poor competitor whose effects can be overcome by comparatively low levels of sulphate. Addition of sodium sulphate (10 mM) to culture medium containing 20 mM chlorate abolished the inhibitory effect (Fig. 2), confirming that the developmental effects were a direct result of chlorate ions acting as competitive inhibitors of macromolecule sulphation.

Chlorate inhibits synthesis of sulphated glycosaminoglycans

To study the effect of chlorate on sulphated GAGs, kidney rudiments were cultured in medium supplemented with [³H]glucosamine to label GAG chains, and with varying concentrations of sodium chlorate. GAGs were released by protease digestion and were analysed by anion-exchange chromatography. Typical FPLC chromatography traces are illustrated in Fig. 3A, while the dose/response curve is shown in Fig. 3B. The results show that chlorate treatment reduces GAG sulphation in a dose-dependent manner, sulphation being almost abolished by 20-30 mM chlorate. Also shown in Fig. 3B is a quantitative analysis of the effect of different chlorate concentrations on ureteric bud development, assessed by counting the number of branch tips existing after 64 hours of incubation. There is a striking correlation between the morphological and biochemical effects of chlorate.

While they inhibited sulphation of newly synthesized glycan chains very effectively, chlorate ions reduced but did not quite
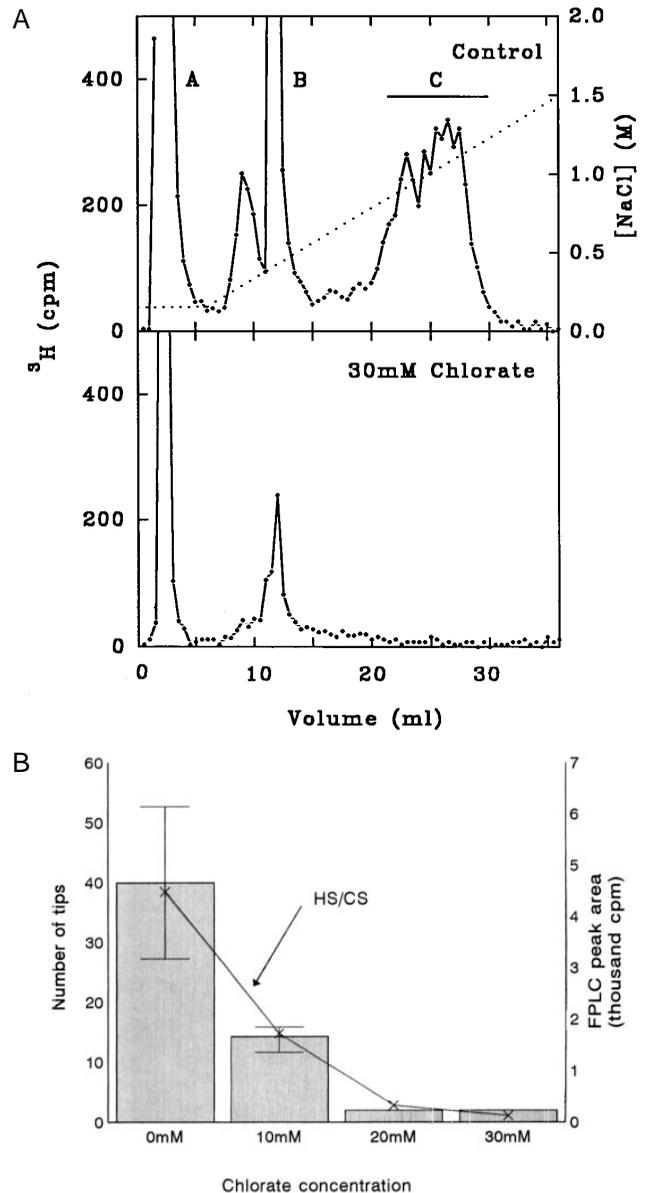
Fig. 3. (A) Typical FPLC Mono-Q anion-exchange chromatography traces of GAG extracted by protease treatment from kidney rudiments cultured in the presence of [³H]glucosamine, with (bottom panel) or without (top panel) 30mM chlorate. The three main peaks are: A, glycoproteins and unincorporated label; B, hyaluronate; C, sulphated proteoglycans. The broad sulphated GAG peak shows a partial resolution of earlier-eluting HS from the later-eluting CS/DS. Treatment with 30mM chlorate abolishes the sulphated glycosaminoglycan peak. (B) Dose-response curve for chlorate. Bars represent the average (± s.d.) number of branch tips formed by ureteric bud, cultured for 64 hours in particular concentrations of chlorate. The line indicates combined heparan/chondroitin sulphate FPLC peak heights, obtained from [³H]glucosamine-labelled kidney rudiments growing at the same chlorate concentrations.

eliminate immunochemically detectable heparan sulphate proteoglycan (HSPG) and chondroitin sulphate proteoglycan (CSPG) (Fig. 4A,B). Production and location of syndecan-1 core protein was apparently unaffected by the chlorate treatment (Fig. 4C).

Combined heparitinase and chondroitinase ABC mimic the effects of chlorate

The specific enzymes heparitinase and chondroitinase ABC were used to confirm the effects of sulphated GAG loss on ureteric bud development, and to identify whether normal morphogenesis of the ureteric bud requires HSPG, CSPG, or both. Immunohistochemical examination of cultures treated with each enzyme showed that heparitinase degraded most of the HS to yield 'heparitinase stubs', i.e. minimal protein-linked fragments displaying a non-reducing terminal unsaturated hexuronate residue (detected by mAb 3G10; David et al., 1992) (Fig. 4D); there was no apparent effect on CS. Chondroitinase ABC degraded CS/DS (CS loss being detected by mAb CS-56) but had very little heparitinase-like activity (detected by mAb 3G10). The effects of the enzymes on ureteric bud development are shown in Fig. 5. Two units of each enzyme alone (0.67 U/ml) resulted in a partial inhibition of ureteric bud development, but a combination of one unit of each (0.33 U/ml) abolished ureteric bud branching completely. As with chlorate, the enzymes had no effect on nephron induction and morphogenesis. The GAG chains of both HSPG and CS/DSPG (chondroitin/dermatan sulphate proteoglycan) carbohydrate chains are therefore required for normal morphogenesis and growth of the ureteric bud, and the effects of chlorate on morphogenesis can be explained by its inhibition of GAG sulphation. Because both chlorate and glycanases produce the same developmental defects, it is extremely unlikely that these defects are due to unexpected side-effects of the individual treatments.

## Effect of chlorate on DNA synthesis

Growth, as well as branching, of the ureteric bud appeared to be inhibited by chlorate (compare Fig. 1A and 1E), suggesting that the loss of sulphated proteoglycan may result in inhibition of cell cycling. To test this hypothesis, kidney rudiments cultured for 20 hours in medium with or without chlorate were supplied, for a 4-hour period, with the thymidine analogue, BrdU, then fixed and stained immunocytochemically for BrdU incorporation. Those cultured in standard medium showed BrdU incorporation (and thus DNA synthesis) in many cells of both the ureteric bud and the nephrogenic mesenchyme surrounding it (Fig. 6A). Those cultured in 20 mM chlorate also showed strong BrdU incorporation in the mesenchyme, but very little in the ureteric bud (Fig. 6B).

To determine whether inhibition of cell cycling alone can reproduce the aberrant morphology induced by chlorate ions, kidney rudiments were cultured in standard medium enriched with 500 nM methotrexate for 24 hours, a treatment sufficient to abolish DNA synthesis completely (Chabner and Young, 1973). Normal growth of the ureteric bud was prevented, but distinct 'bumps' appeared on the surface of the bud in similar numbers to true branch tips formed in control cultures (Fig. 6C). The simplest explanation for these 'bumps' was that they represented the first stage in new branch formation. In the absence of any cytochemical markers for normal branch initiation we could not test this hypothesis directly. Our results therefore suggested that the morphogenetic process of branch intitiation was not dependent on DNA synthesis. Thus there may be two effects of sulphated GAG deprivation, one on morphogenesis and one on cell cycling in the ureteric bud.

Sulphated GAG deprivation does not inhibit expression of c-met and c-ros in ureteric bud

During normal development, the ureteric bud epithelia and
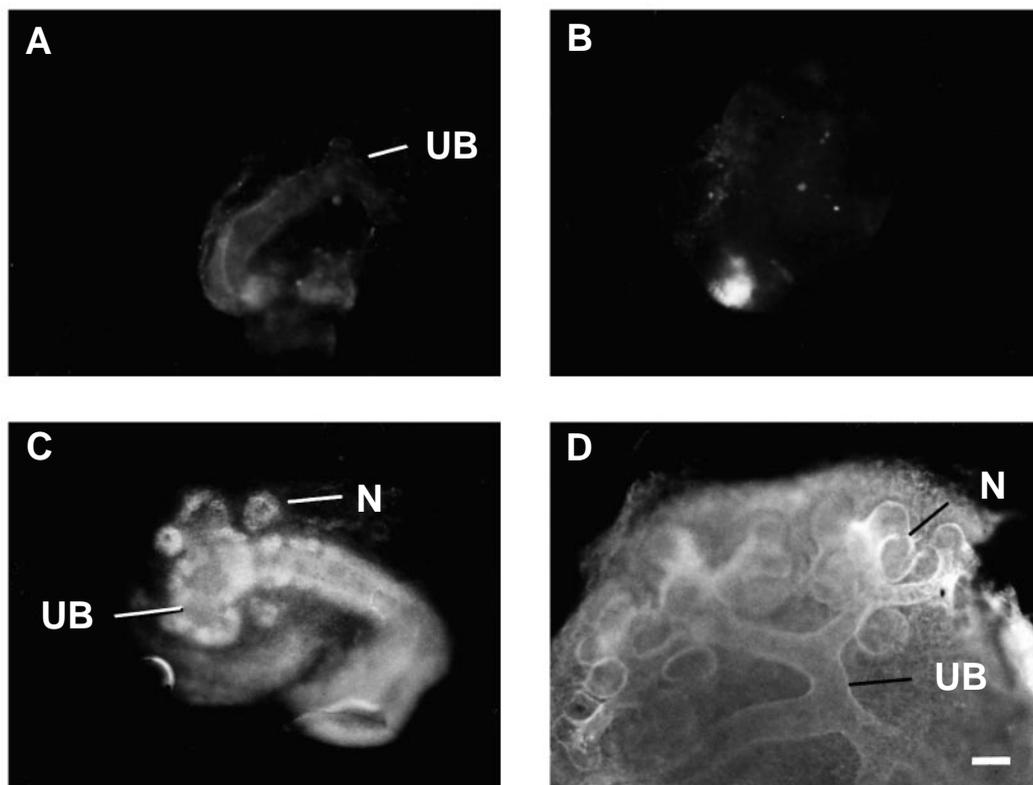
Fig. 4. (A) Anti-heparan sulphate staining of kidney rudiments treated with 30 mM chlorate (compare with Fig. 1B). Expression in the mesenchyme has been almost eliminated, though the basement membrane of the ureteric bud (UB) still contains some antigen, especially at its base (where it once joined the Wolffian duct). (B) Anti-chondroitin sulphate staining of kidney rudiments treated with 30 mM chlorate (compare with Fig. 1C). Expression of chondroitin sulphate is almost eliminated by chlorate treatment except at the very base of the ureteric bud (arrow). (C) Anti-syndecan-1 core protein staining of kidney rudiments treated with 30 mM chlorate (compare with Fig. 1D). Expression of the core protein is apparently unaffected in both ureteric bud (UB) and developing nephrons (N). (D) Anti-'heparitinase stub' (mAb 3G10) staining of kidney rudiments treated with 0.67 U/ml heparitinase; the antibody binds to the glycan 'stubs' created by the action of heparitinase on HSPGs. Scale bar, 100 µm.
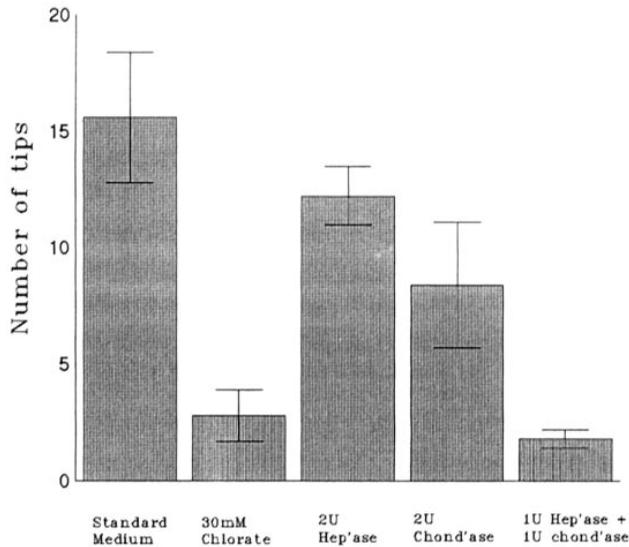
Fig. 5. (A) Heparitinase and chondroitinase ABC mimic chlorate. The effects of chlorate and the enzymes heparitinase and chondroitinase ABC on ureteric bud development were quantified by counting ureteric bud tips; each enzyme can partially mimic the effect of chlorate, while the combination of both enzymes mimics it completely.

developing nephrons both express the c-met receptor tyrosine kinase, the ligand for which, hepatocyte growth factor, is important to renal growth (Sonnenberg et al., 1993; Santos et al., 1994). To determine whether c-met expression was altered by GAG-deprivation, kidneys cultured in standard medium or 30 mM chlorate for 48 hours were stained using a polyclonal antibody against murine c-met. Expression of the c-met protein was similar in both media (Fig. 7), being weak in mesenchyme cells and strong in developing nephrons and ureteric bud; this is the pattern described by Woolf et al. (1995).

Another receptor tyrosine kinase, c-ros, is expressed by normal developing kidneys (Tessarollo et al., 1992). Examination of its expression by in situ hybridisation demonstrates that the ureteric-bud-only pattern of normal kidneys (Tessarollo et al., 1992) is maintained in those deprived of GAGs (Fig. 6E).

Hepatocyte Growth Factor restores growth but not branching of sulphated GAG-deprived ureteric buds

Sulphated GAGs participate in the binding of several growth factors, and are essential for the function of some, such as bFGF (Yayon et al., 1991; Rapraeger et al., 1991). Disruption of normal GAG structure may therefore inhibit cell cycling in the ureteric bud by failure of growth factor signalling. To test this hypothesis, kidney rudiments cultured in chlorate (30 mM) were supplied with exogenous growth factors, to determine whether a high concentration of one of them would compensate for severe reduction in available sulphated GAG. Of the growth factors tested (HGF, bFGF, EGF, TGFβ1), only human HGF produced an effect. Ureteric buds treated with 100 ng/ml HGF in the presence of chlorate continued to elongate but showed no evidence of branching, producing the aberrant morphology shown in Fig. 6E. Addition of HGF to chlorate-treated cultures elevated ureteric bud BrdU incorporation slightly, but

did not restore it to levels found in standard medium, a result compatible with the observation that the length of HGF-extended chlorate-treated ureteric bud is much less than the total length of branches formed in standard medium (compare Figs 1A, 6E). The fact that ureteric bud growth is partially restored, without restoration of branching, again suggests that ureteric bud development consists of distinct growth and morphogenetic components, both of which are inhibited by chlorate.

A protein kinase C activator restores branch intitation but not growth of sulphated GAG-deprived ureteric bud

To investigate whether activation of a cell-signalling pathway could rescue ureteric buds from the morphogenetic effects of chlorate, we treated kidney rudiments with a variety of second messenger modulators. The effects of the protein kinase A activator, dibutyryl cyclic AMP, and the protein kinase C activator, phorbol 12-myristate acetate (PMA), were assessed on kidney rudiments grown in standard or chlorate medium. To minimise the effects of non-specific toxicity, these experiments were performed for only 12-18 hours of culture rather than 48-96 hours; consequently the number of ureteric bud branches was much smaller than in other experiments, even in the controls (approx. 5 branch tips rather than >30).

Dibutyryl cAMP (1 mM) neither reproduced nor inhibited the chlorate effect. In contrast, continuous presence (0-18 hours) of the protein kinase C activator, PMA, resulted in the production of protrusions on the surface of an otherwise non-branching ureteric bud (Fig. 6F). The 'bumps' (Fig. 8A,B), which were very similar to those produced by methotrexate treatment of kidney rudiments (Fig. 6C), failed to extend into true branches. A dose-response curve for the PMA effect is shown in Fig. 9; at its most effective concentration (50 ng/ml), PMA produced almost as many 'bumps' as there were true branch tips in the

Fig. 6. (A) Incorporation of BrdU by kidneys cultured in standard medium; cells in both the ureteric bud (UB) and the metanephrogenic mesenchyme (M) show strong incorporation (arrowheads indicate examples). The ureteric bud basement membrane is detected by anti-laminin staining (bm).
(B) Incorporation of BrdU by kidneys cultured in 30 mM chlorate; while DNA synthesis (arrowheads) continues in the mesenchyme (M), that in the ureteric bud (UB) has almost been eliminated (open arrows show the few ureteric bud nuclei that have incorporated BrdU). (C) Methotrexate treatment of kidneys prevents growth, but allows the ureteric bud to produce bumps as if branch initiation is still taking place (arrows; anti-laminin stain). (D) Expression of c-ros mRNA in chlorate-treated kidneys; the gene is transcribed throughout the ureteric bud (UB). (E) Effect of 100 ng/ml HGF on chlorate-treated kidneys; the arms of the ureteric bud (which was T shaped at the start of the experiment, like that shown in D) have elongated without branching over the course of 7 days (arrows). Some nephrons have fused with the ureteric bud (arrowheads), an event which can also take place in chlorate alone (anti-laminin stain).
(F) Effect of 50 ng/ml PMA on chlorate-treated kidneys; the arms of the ureteric bud have not elongated, but have produced bumps suggestive of branch initiation (arrows; anti-laminin stain).
(G,H) Combined effect of HGF and PMA on chlorate-treated kidneys: ureteric buds of kidneys cultured in chlorate alone show little growth or branching (G), while those in chlorate supplemented with 50 ng/ml PMA and 100 ng/ml HGF show both growth and branching (H) (anti-laminin stain). Scale bars, 100 μm.
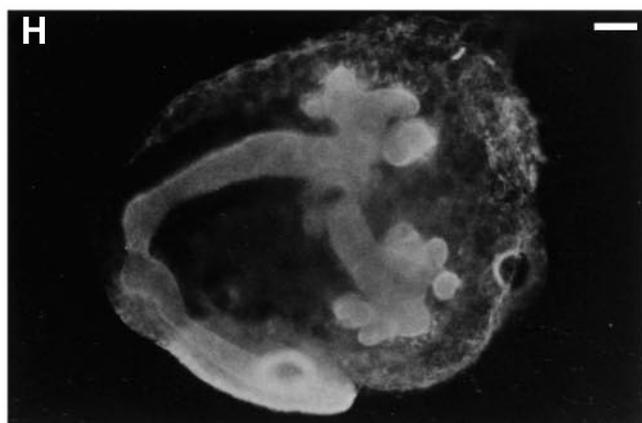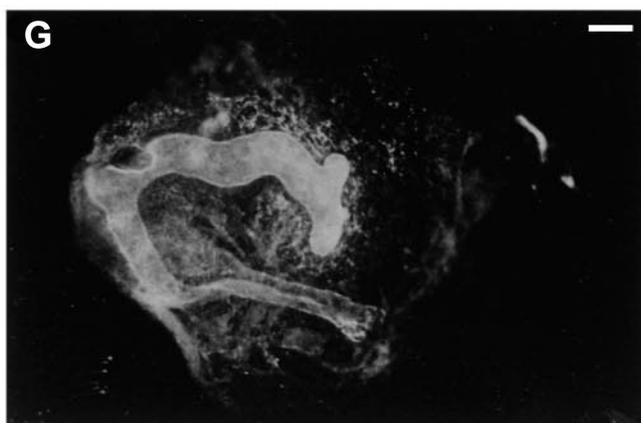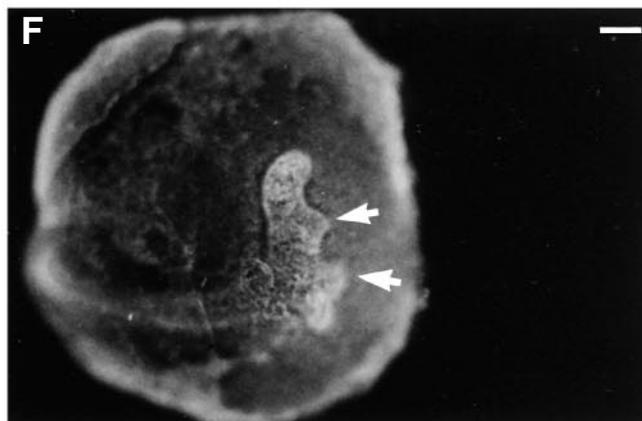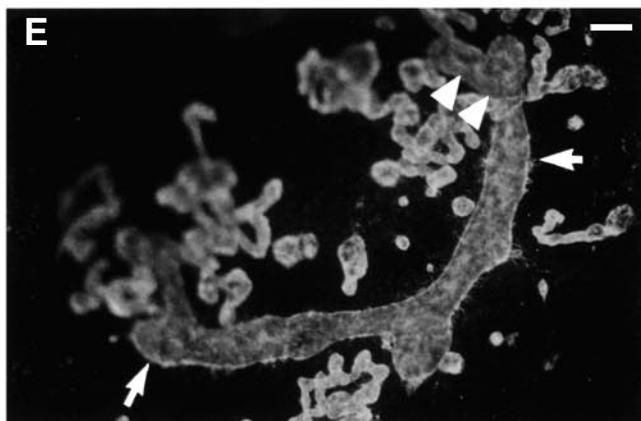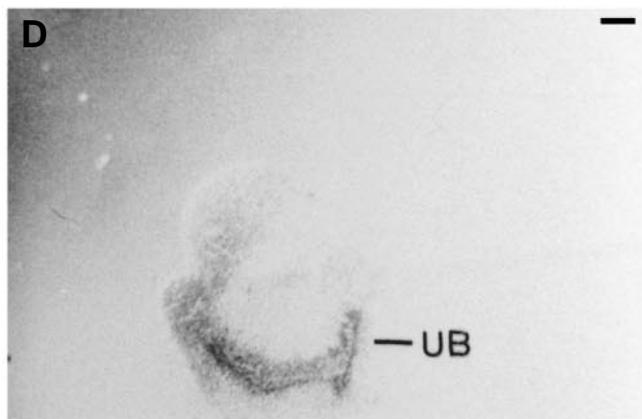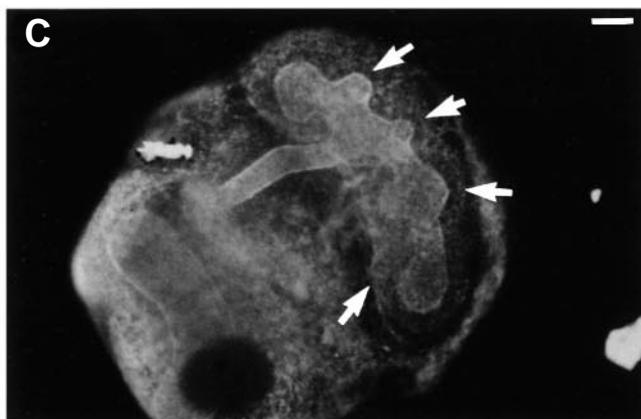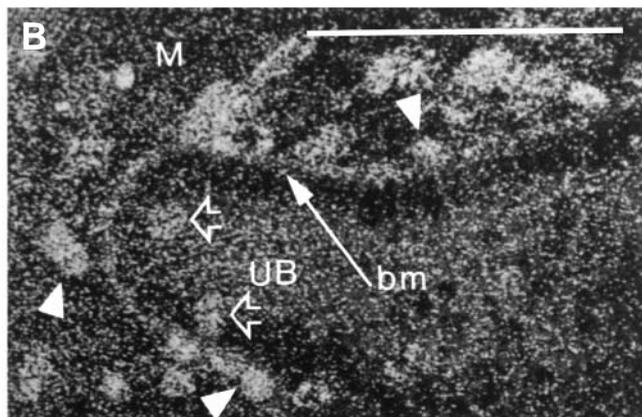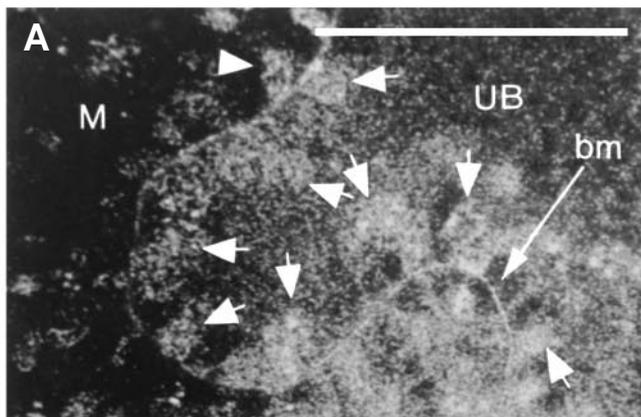
controls, again suggesting that production of bumps and branches is related. While PMA treatment restored this limited morphogenetic activity to chlorate-blocked ureteric buds, it did not restore cell cycling (assessed by BrdU incorporation).

PMA is a short-term activator but a long-term inhibitor of PKC; the 18-hour treatments described above are long enough eventually to inhibit the enzyme after an intitial activation (Santos et al., 1993). The morphogenetic effect of PMA could therefore have been mediated by the PKC-inhibiting phase of its action, or a combination of both PKC activation and PKC inhibition. Short term (0-1 hour) treatment of kidney rudiments in 30 mM chlorate with 50 ng/ml PMA, followed by 17 hours in PMA-free medium, failed to produce the ureteric bud 'bumps' elicited by continuous (0-18 hours) presence of PMA. In an attempt to investigate this further, we applied stau-rosporine, an inhibitor of protein kinases including PKC (at 5-50 nM; Santos et al., 1993), to chlorate-treated and control kidney rudiments. At 5-10 nM, a concentration that elicits morphogenesis in collecting duct-derived MDCK cells (Santos et al., 1993), the drug failed to induce bump formation by sulphated GAG-deprived ureteric buds and it allowed control ureteric buds to develop normally. At 50 nM, staurosporine still failed to induce bump formation, but became toxic to normal ureteric bud growth, inhibiting it irreversibly. The inability of staurosporine to induce bumps even at non-toxic concentrations (5-10 nM) suggests that long term PMA treatment did not act simply through inhibition of PKC, but that the activation phase was also important.

## Combined HGF and PMA stimulates growth and branching of GAG-deprived ureteric bud

Since different aspects of ureteric bud development were restored by HGF and PMA individually, we tested their combined ability to negate chlorate treatment. In the continuous (0-18 hours) presence of both reagents (HGF 100 ng/ml, PMA 50 ng/ml) the ureteric bud of chlorate- (30 mM) treated kidneys both branched and grew. However, the morphology of branching was not quite normal, branches being short and abnormally close together (Fig. 6G,H).

DISCUSSION

We have investigated the function of sulphated glycosaminoglycans in developing kidney and have found that their loss causes a striking inhibition of ureteric bud development and a reduction in the number of nephrons. The development of nephrons that do form is apparently normal.

Strongly elevated HSPG and CSPG synthesis is one of the earliest events in nephro-

genesis, which suggests an important functional role for the molecules (Vainio et al., 1989, 1992; David et al., 1992; Davies and Garrod, 1995). Interference with sulphated GAG production might therefore be expected to inhibit critical developmental events, if not at the early stages of the mesenchyme-epithelium transition, then during tubule elongation, morphogenesis, or fusion with the ureteric bud. We were therefore suprised to discover that, once they have been induced, developing nephrons seem indifferent to almost complete loss of their heparan and chondroitin/dermatan sulphate chains, and develop apparently anatomically normally (nephron function could not be tested in this system). The only
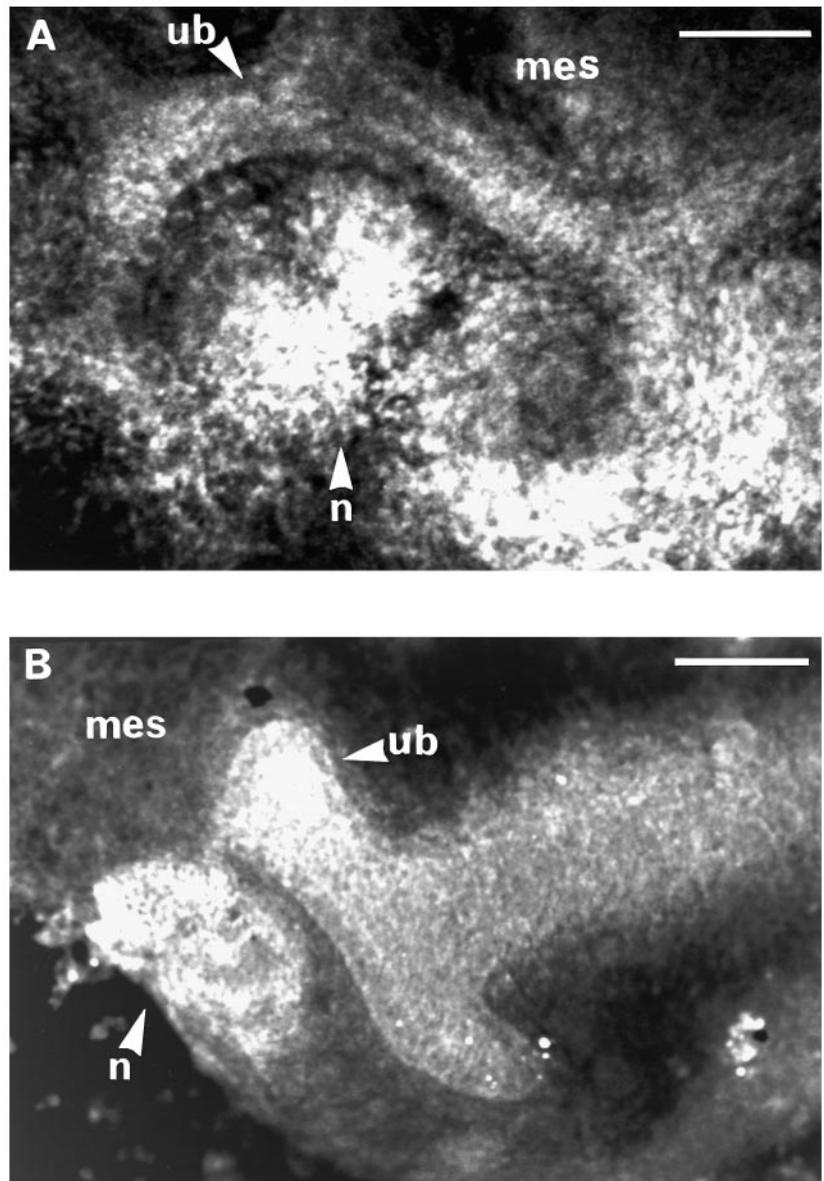
Fig. 7. Expression of c-met, the HGF receptor tyrosine kinase, by kidneys in standard medium or 30 mM chlorate. (A) Kidney rudiment cultured in standard medium and stained for murine c-met. The antibody recognises mesenchymal cells (mes) weakly, and ureteric bud (ub) and developing nephrons (n) strongly. (B) Kidney rudiment cultured in 30mM chlorate and stained for murine c-met. Again, the mesenchyme (mes) stains weakly and the ureteric bud (ub) and developing nephron (n) strongly. Scale bars, 100 μm.

effect of sulphated GAG-deprivation on nephrogenesis was a reduction in the numbers of nephrons formed. This might be due to a requirement for sulphated GAGs in the initiation of nephron development, or might alternatively reflect the failure of the GAG-deprived inducing tissue (ureteric bud) to ramify through the nephrogenic mesenchyme.

Even though sulphated glycosaminoglycan is not required for nephron induction and development, it remains possible that the proteoglycan core is needed. The syndecan-1 core protein was synthesised and located normally even when its glycan chains were unsulphated, so any purely protein-mediated interactions would have been maintained. However, proteoglycan functions known to be mediated by the carbohydrate chains, such as growth factor binding (for review see Gallagher, 1994), are unlikely to be necessary for nephron differentiation. If such interactions occur at all, they must be part of a system with sufficient redundancy to allow development to proceed despite the loss of one component (sulphated GAGs); in this context it is interesting to note that attempts to prevent nephron differentiation by inhibition of several other potential morphoregulatory molecules, such as NCAM and E-cadherin, have failed (Klein et al., 1988; Vestweber et al., 1985).

In contrast to nephron development, that of ureteric bud was affected greatly by loss of sulphated GAGs. Blockade of normal GAG maturation by the sulphation inhibitor, sodium chlorate (Farley et al., 1978; Rapraeger et al., 1991), prevented ureteric bud growth and branching in a dose-dependent manner. Enzymatic degradation of heparan and chondroitin/dermatan sulphates produced apparently identical effects, although the enzymes had to be used together to inhibit branching completely. As well as confirming the specificity of the chlorate effect, these results indicate that both HSPG and CS/DSPG are important to ureteric bud development.

Blockade of DNA synthesis with methotrexate inhibits growth but not branch initiation in the ureteric bud. By contrast, inhibition of GAG sulphation prevents both growth and branch initiation. This indicates that GAGs must be required for a morphogenetic process separate from simple cell cycling. By testing the ability of growth factors and protein kinase activators to rescue ureteric buds from the effects of chlorate, we have confirmed that growth and morphogenesis are controlled by different pathways.

These results reveal several interesting similarities between arborisation of ureteric bud and that of submandibular gland epithelium. Submandibular glands contain HS/CSPGs in their basement membranes, degradation of which inhibits both branching and DNA synthesis (Thompson and Spooner, 1982; Nakanishi et al., 1993). However, inhibition of DNA synthesis alone (by X-irradiation or aphidicolin) does not prevent branch initiation (Nakanishi et al., 1987), suggesting that in this system too sulphated GAGs are required for separate morphogenetic and mitogenic signals. The similarities in GAG-requirement between ureteric bud and submandibular gland are especially striking given great apparent difference in the mechanism of branching;
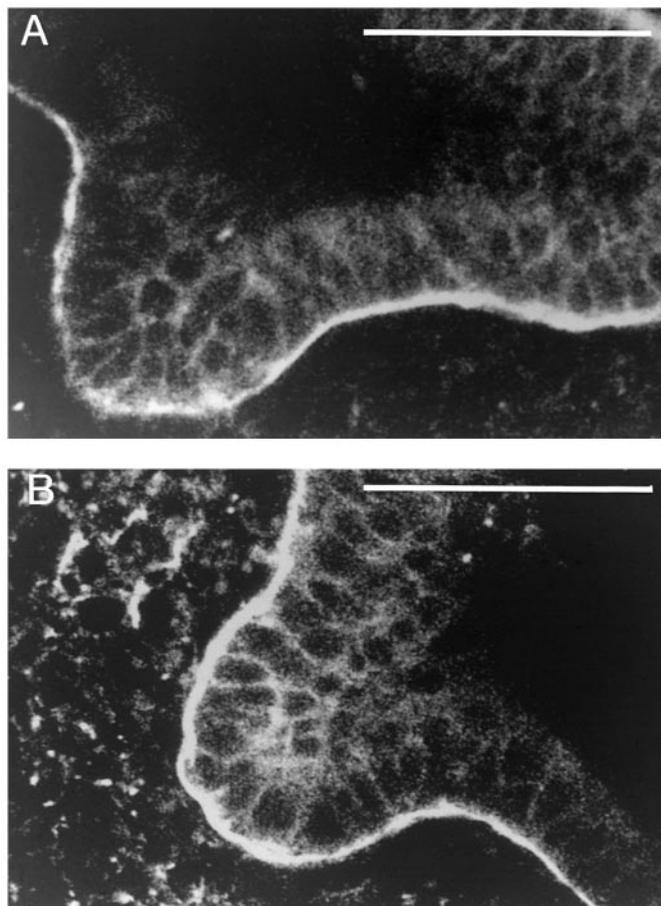
Fig. 8. Comparison of ureteric bud 'bumps' induced by PMA with those seen during normal ureteric bud bifurcation. (**A**) Confocal image of the 'bump' formed by a uretric bud beginning its first bifurcation, in a kidney rudiment cultured in standard medium for 6 hours and stained for α6-integrin. (B) Confocal image of a ureteric bud 'bump' induced by PMA treatment of a kidney rudiment incubated in medium supplemented with 30mM chlorate, again stained for α6-integrin. The two 'bumps' are similar in size, shape and number of cells. Scale bars, 100 μm.
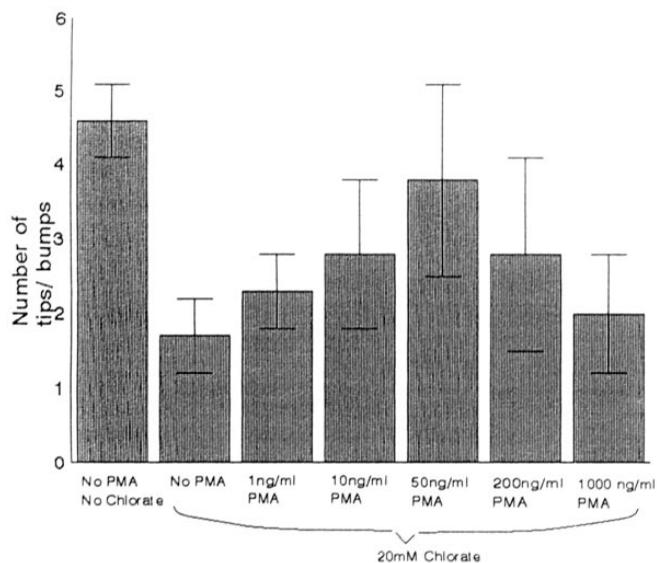
Fig. 9. Dose-response for PMA. Each bar represents the number of true branches or 'bumps' (see main text) formed by ureteric buds cultured in 30 mM chlorate with different concentrations of added PMA. At its most effective concentration (50 ng/ml), PMA elicits approximately as many 'bumps' as there were true branches in control cultures.

ureteric bud branches originate as bumps pushing out from a smooth tube (Fig. 8), while salivary gland branches form when the rounded tips of growing tubes are cleaved by an extracellular matrix tourniquet (see Nakanishi and Ishi, 1989, for review).

Growth of chlorate-blocked ureteric buds can be restored by treatment with high concentrations of hepatocyte growth factor (HGF). HGF is an unusually large (80K) growth factor, with a 38% sequence identity to plasminogen, which is synthesised as a single polypeptide chain and cleaved extracellularly to yield an a/β chain heterodimeric protein (for review see Mizuno and Nakamura, 1993). It binds with high affinity (K_d=2-3×10^{-11} M) to the receptor tyrosine kinase, c-met, another dimeric protein produced by proteolysis of a single polypeptide chain (Higuchi et al., 1992; Weidner et al., 1993). In developing kidney, mesenchymal cells synthesise active HGF, both mesenchymal and epithelial cells transcribe c-met, and inhibition of their interaction using anti-HGF antibodies reduces the overall growth of kidney rudiments in culture (Sonnenburg et al., 1993; Santos et al., 1994; Woolf et al., 1995). Because c-met is expressed by the mesenchyme as well as the epithelia, it is not certain that the effects of sulphated GAGs and HGF act directly on the ureteric bud epithelia; an alternative possibility is a primary action on the mesenchyme which modulates its ability to induce ureteric bud development (and also perhaps the number of nephrons that the mesenchyme can form).

The action of HGF is potentiated by heparan sulphate chains, to which the a chain of HGF binds (K_d=2-5x10^{-10} M) under physiological conditions (Naka et al., 1993; Lyon et al., 1994; Lyon and Gallagher, 1994). By binding HGF, cell surface heparan sulphate may act through raising its local concentration and thus make interaction with the c-met receptor more probable. Such a mechanism could provide one possible explanation of why we were able to reverse partially the effect of sulphated GAG deprivation by using very high concentrations of exogenous HGF (100 ng/ml), which would make the GAG-mediated local concentration system less important (for comparison, HGF affects cells with intact GAGs at only 2 ng/ml; Montesano et al., 1991b).

HGF acts as a mitogen and as a stimulator of cell movement, depending on cell type. In cells such as hepatocytes it simply promotes cell cycling (Nakamura et al., 1984, 1986), but in sub-confluent cultures of MDCK cells it promotes loss of contact between and dispersal of cells, hence its synonym Scatter Factor (Stoker et al., 1987; Weidner et al., 1991). While monolayer cultures of MDCK cells respond to HGF by scattering, provision of a three-dimensional collagen matrix alters the response to one more typical of normal morphogenesis; MDCK cells grown in collagen form cysts, which grow, elongate and branch in response to HGF (Montesano et al., 1991b). Because MDCK cells are thought to have arisen from collecting duct, it has been assumed that the morphogenetic effect of HGF on MDCK cell cysts in culture reflects a role in ureteric bud/collecting duct branching in vivo (Montesano et al., 1991a,b; Santos et al., 1993). However, while we have shown that HGF promotes growth of sulphated GAG-deprived ureteric buds, we found no evidence that it promotes branching. There is a further difference between the systems, in that the earliest response of MDCK cell cysts to activation of growth and branching by HGF is the production of fine cell processes (Montesano et al., 1991a,b; Santos et al., 1993), but in developing kidney, processes form when growth and branching are <u>inhibited</u> by GAG deprivation.

HGF may act differently in the two systems, perhaps because of the unusual properties of the MDCK line (which has lost the ability of primary kidney epithelial cells to form tubes without addition of HGF; Taub et al., 1990). Alternatively, even the large HGF concentrations used here may be insufficient to promote branching in the absence of sulphated GAGs.

Branch initiation (formation of 'bumps') in chlorate-blocked ureteric buds is stimulated by 18-hour treatment with PMA, but ureteric bud growth is not. PMA, a phorbol ester, is a modulator of protein kinase C (Castagina et al., 1982), suggesting that while cell cycling in this system is regulated by protein tyrosine phosphorylation (via HGF/c-met), branching morphogenesis is controlled by protein serine/threonine phosphorylation (via PKC). PKC also regulates morphogenesis in the MDCK cyst system, although the effects of PKC modulators are rather different to those we describe; in the MDCK cysts, PKC inhibition by either staurosporine or 18-hour PMA treatment mimics HGF-mediated induction of cell processes (Santos et al., 1993).

Our finding that PMA induces limited morphogenesis without apparent growth in the ureteric bud has two implications; it confirms that ureteric bud development involves two distinct processes – growth and branching – and suggests that each requires sulphated GAGs as part of a signalling pathway (rather than directly for cell adhesion, for example). The precise effect of PKC modulation on the ureteric bud cells, which enables groups of them to push out as distinct 'bumps' from an otherwise smooth tubule, remains to be discovered. In principle, this morphogenesis could be accomplished internally, for example by contraction of the apical cytoskeleton to produce wedge-shaped cells, or by altering production of, destruction of or adhesion to the external basement membrane. The fact that some groups of cells participate in PMA-induced 'bump' formation while intervening ones do not, suggests the existence of local cell-cell interactions which can regulate this choice.

Treatment with a combination of HGF and PMA restores both growth and branching to chlorate-blocked ureteric buds, though the resulting morphology is not precisely normal. The abnormal morphology may indicate a third function for sulphated GAGs in this system, connected with regulation of branch patterns, or it may reflect an imbalance between experimental growth and branching stimuli. In any case, our discovery that manipulation of just two signalling effectors can alter the morphology of a branching epithelium implies that the evolution of new organ shapes from old may require few genetic mutations.

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