Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney

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SUMMARY

Branching morphogenesis of epithelium is a common and important feature of organogenesis; it is, for example, responsible for development of renal collecting ducts, lung airways, milk ducts of mammary glands and seminal ducts of the prostate. In each case, epithelial development is controlled by a variety of mesenchyme-derived molecules, both soluble (e.g. growth factors) and insoluble (e.g. extracellular matrix). Little is known about how these varied influences are integrated to produce a coherent morphogenetic response, but integration is likely to be achieved at least partly by cytoplasmic signal transduction networks. Work in other systems (Drosophila tracheae, MDCK models) suggests that the mitogen-activated protein (MAP) kinase pathway might be important to epithelial branching. We have investigated the role of the MAP kinase pathway in one of the best characterised mammalian examples of branching morphogenesis, the ureteric bud of the metanephric kidney. We find that Erk MAP kinase is normally active in ureteric bud, and that inhibiting Erk activation with the MAP kinase kinase inhibitor, PD98059, reversibly inhibits branching in a dose-dependent manner, while allowing tubule elongation to continue. When Erk activation is inhibited, ureteric bud tips show less cell proliferation than controls and they also produce fewer laminin-rich processes penetrating the mesenchyme and fail to show the strong concentration of apical actin filaments typical of controls; apoptosis and expression of Ret and Ros, are, however, normal. The activity of the Erk MAP kinase pathway is dependent on at least two known regulators of ureteric bud branching; the GDNF-Ret signalling system and sulphated glycosaminoglycans. MAP kinase is therefore essential for normal branching morphogenesis of the ureteric bud, and lies downstream of significant extracellular regulators of ureteric bud development.

Key words: Erk, kidney, MAP kinase, Branching, Mouse

INTRODUCTION

Branching morphogenesis of tubular epithelium is a common and important feature of vertebrate organogenesis; examples of tissues formed in this manner include collecting ducts of the kidney, airways of the lung, milk ducts of the mammary gland and seminal ducts of the prostate. In all cases, development of the epithelium is regulated by a large number of molecules produced by the surrounding mesenchyme; these include growth factors, proteases and components of the extracellular matrix (Hieda and Nakanishi, 1997; Davies and Davey, 1999; Warburton et al., 2000). Cells of the epithelium have to integrate these signals to produce a coherent morphogenetic response, and while the process of integration is little understood, much of it probably takes place in cytoplasmic signal transduction networks. To identify candidate networks for integrating and controlling branching morphogenesis, we have examined the role of a major signal transduction pathway, the mitogen-activated protein (MAP) kinase pathway, in what is arguably the best understood mammalian example of epithelial branching: the developing urinary collecting duct.

The collecting duct system of the kidney derives from the ureteric bud, an initially unbranched outgrowth of the Wolffian duct (Davies and Davey 1999). The ureteric bud develops in response to glial cell line-derived neurotrophic factor (GDNF), which is secreted by the nearby metanephric mesenchyme; this system is so powerful that Gdnf−/− transgenic mice fail to form any ureteric buds at all (Pichel et al., 1996; Moore et al., 1996; Sainio et al., 1997). The Ret receptor tyrosine kinase that binds GDNF can also be activated by other members of the GDNF family, such as neurturin, persephin and artemin (Balogh et al., 2000). Neurturin can also elicit bud formation in culture assays (Davies et al., 1999), but as this molecule is naturally synthesised by the bud itself, it is unlikely to be an in vivo inducer.

Once inside the metanephric mesenchyme, the ureteric bud begins to arborise to form the collecting duct tree. GDNF is still required for this, and both neurturin and persephin can promote branching in culture (Sainio et al., 1997; Milbrandt et al., 1998; Davies et al., 1999). Hepatocyte growth factor
is active in the tips of developing branches, and its activity is the MAP kinase pathway (Fig. 1) (Huang and Erikson, 1996). From bone morphogenetic protein (BMP) 2. In other systems, kinase A pathway in transducing branch-inhibiting signals Gupta et al. (Gupta et al., 1999) has implicated the protein transduction in ureteric bud arborisation, although the work of actual morphogenesis. So far, little is known about signal integration and subsequent control of gene expression and transduction networks in the ureteric bud cells for processing, molecules are passed, via their receptors, to cytoplasmic signal transduction in normally developing ureteric bud, that its activity is necessary for branching morphogenesis to take place, and that the pathway is regulated by GDNF and also requires glycosaminoglycans.

MATERIALS AND METHODS

Kidney culture

Kidney rudiments were isolated from MF1 mouse embryos at embryonic day (E)11.5 (morning of discovery of vaginal plug was taken to be E0.5) and were cultured on track-etched polycarbonate filters at the medium/gas interface for 2-4 days, according to methods we have described elsewhere (Davies, 1994). For experimental treatments, the medium (Eagle’s MEM with Earle’s salts (Sigma, M5650) with 10% heat-inactivated newborn calf serum, penicillin and streptomycin) was supplemented with one or more of the following: 2'-amino-3'-methoxyflavone (PD98059; Calbiochem) at 5-50 μM, NaClO2 (Merck) at 30mM (Davies et al., 1995), 10 ng/ml BMP2, or function-blocking anti-GDNF antibody (R&D systems) at 10 μg/ml (Vega et al., 1996; Davies et al., 1999). Lung rudiments were isolated from mice at E10.5 and E11.5; for ureteric bud/lung recombinants, organ rudiments were treated in trypsin solution for 3 minutes, then dissected into their epithelial and mesenchymal components between fine needles. Epithelium and mesenchymes were recombined by surrounding one or two epithelial rudiments with several fragments of mesenchyme on polycarbonate filters. For all lung experiments (whether or not they involved tissue recombination), culture medium was the renal culture medium supplemented with 10 ng/ml GDNF (Sainio et al., 1997). Hanging drop culture of ureteric buds was performed according to the method of Sainio et al. (Sainio et al., 1997); ureteric buds were isolated from kidneys by manual dissection after incubation of kidneys in trypsin for 20 minutes at 37°C. They were examined at high power to ensure that their outlines were smooth (basement membrane) rather than ragged (adhering mesenchyme), and were then transferred to 40 µl drops of medium with or without 50 ng/ml GDNF and 25 μM PD98059 hanging from the lid of a 3 cm petri dish (CellStar). They were incubated for 40 hours, then photographed using a Zeiss dissecting microscope.

Immunohistochemistry

Immunohistochemistry was carried out using procedures and antibodies we have described before (Davies et al., 1995; Davies et al., 1999). For staining with anti-calbindin-D-28K, a specific marker for ureteric bud and developing collecting ducts (Davies, 1994), cultures were fixed in –20°C methanol, still attached to their filters, for 15 minutes then washed in PBS (phosphate-buffered saline 0.1 M, pH 7.4). They were stained in primary antibody (Sigma C8666 at 1/100) for 3 hours at 37°C, washed in PBS, incubated with 1/100-1/200 FITC or TRITC-conjugated secondary antibody (Sigma) for 2 hours at 37°C, washed in PBS, mounted in glycerol/PBS and viewed under a Leitz epifluorescence microscope. For staining with anti-Ret (Santa Cruz sc-167) and anti-Ros (Santa Cruz sc-6348), cultures were fixed and washed as above, then incubated in 1% bovine serum

Fig. 1. A schematic of the classical Erk 1/2 MAP kinase pathway, showing typical connections to and from the pathway, and showing the site of action of the inhibitor, PD98059.

Receptor tyrosine kinases

↓

Grb2-Sos

↓

Ras

↓

Raf-1

↓

Mek 1 & 2

PD98059

↓

Erk 1 & 2

Calponin

Myc

Fos

↓

↓

↓

↓
albumin (BSA) 0.1% Triton X-100 in PBS for 1 hour and incubated in primary antibody (1/50 anti-RET, 1/200 anti-Ros) in 1% BSA in PBS at 4°C overnight, then stained with secondary antibody as above. Cultures to be stained with phalloidin were fixed in 4% formaldehyde in PBS overnight at 4°C, washed in PBS then stained overnight at 4°C with 220 ng/ml TRITC-phalloidin (Sigma P1951). After washing they were examined using a Leica confocal microscope.

**BrdU incorporation and propidium iodide staining**

Cell proliferation was studied by adding bromodeoxyuridine (BrdU) to culture medium to a final concentration of 100 μM and incubating cultures for a further 16 hours. Cultures were then fixed overnight in 4% formaldehyde in PBS, washed in PBS, incubated in 0.5 mg/ml trypsin for 20 minutes at 37°C, refixed in 4% formalin for 20 minutes at room temperature and washed in PBS. They were then incubated in a mixture of 95% formamide, 5% 0.15 M trisodium citrate for 1 hour at 70°C, washed in PBS and incubated overnight in 1/1000 anti-aminulin (Sigma Cat L9393) and 1/40 anti-BrdU (Sigma B2531). Cultures were then stained with fluorescent secondary antibodies and examined on a Leica confocal microscope. For quantification of incorporation, images were scanned in a plane bisecting the tubule (at which the apparent diameter of the tubule was greatest), the tissue outside the ureteric bud was masked off (as shown in Fig. 4A,B), and BrdU-positive nuclei were counted within approximately 110 μm (precisely 3.00 arbitrary units on the image analysis system). Apoptosis was assessed by examining nuclear morphology. Cultures incubated for 24 hours with or without 25 μM PD98059 were stained for calbindin D (28 K) as described above, but with 1 mg/ml RNAse A included in the primary antibody solution (to remove RNA before propidium iodide staining) and 0.1 μg/ml propidium iodide included with the secondary antibody. Images were imaged using the confocal microscope and analysed as described above; apoptotic nuclei were identified by their bright, condensed appearance.

**Homogenisation of samples**

Kidney rudiments, either freshly dissected or after culture, were transferred to a 0.1 ml glass homogeniser (Jencons) on ice, and 5 μl of lysis buffer (50 mM Hepes, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1% Aprotinin, 1 mM PMSF, 0.5 mM sodium vanadate) was added per kidney. Kidneys were homogenised with 300 strokes of the homogeniser plunger (we found this thoroughness to be important for consistent results), then the homogenate was centrifuged at 13,000 g for 2 minutes, and the supernatant was analysed by western blotting.

**SDS-PAGE and western blotting**

Proteins from kidney homogenates were separated on a 7.5% SDS-PAGE gel using a BioRad mini protein II system and then transferred (in 20% methanol, 25 mM Tris, 192 mM glycine) to Hybond nitrocellulose membranes (Amersham). Membranes were incubated for 30 minutes in 5% dried skimmed milk in PBS and probed with rabbit anti-Erk1/2 (1/1000; Sigma), with mouse anti-phosphorylated Erk1/2 (1/1000; Sigma) or with anti-RET (0.2 μg/ml; Santa Cruz) in PBS containing 1% milk. The secondary antibodies (Sigma) were used at a 1/20,000 dilution (anti-rabbit HRP) or at a 1/2000 dilution (anti-mouse HRP) or at 1/15000 (anti-Goat HRP). Signals were detected by an ECL plus kit (Amersham, RPN 2132) according to the manufacturer’s recommendation. For all samples, detection of Erk1/2 in any phosphorylation state was used as an homogenisation control to confirm that equal amounts of tissue were being examined on blots used for detection of only phosphorylated Erk1/2. In addition, a BioRad protein assay was used to compare total protein levels within samples.

**BioRad protein assay**

The amount of protein in various samples was compared to a standard curve of BSA (1-25 μg/ml) in TGMED K buffer (25 mM Tris, 10% glycerol, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 0.2 mM PMSF). Samples diluted in TGMED K buffer (0.8 ml) were added to 0.2 ml of concentrated BioRad reagent and thoroughly mixed. The samples were left at room temperature for 15 minutes before being read at 595 nm in a Cecil 2000 series spectrophotometer.

**RESULTS**

**Erk1/2 MAP kinase is normally active in both ureteric bud and mesenchymal cells**

For Erk MAP kinases to play a role in regulating branching morphogenesis of the ureteric bud, they must normally be expressed and active in that tissue. To establish this, we used the technique of western blotting with antibodies that recognise only the phosphorylated forms of Erk1 and Erk2 (Yung et al., 1997). Active (phosphorylated) Erks were detected in homogenates of complete developing kidneys freshly isolated from E11 mouse embryos (data not shown), and also in separate homogenates of each of the two constituent tissues of these kidneys, the ureteric bud and metanephric mesenchyme (Fig. 2). Both Erk 1 (44 kDa) and Erk 2 (42 kDa) were phosphorylated, but the Erk2 signal was much stronger in both tissues.

**Activity of the MAPK pathway is required for branching morphogenesis of the ureteric bud**

To assess the requirement for Erk MAP kinase activity in ureteric bud morphogenesis, we used the drug PD98059 (2′, amino-3′-methoxylavone), a specific inhibitor of the Mek MAP kinase kinase that activates Erk in the classical MAP kinase cascade (see Fig. 1). This inhibitor was used at concentrations ranging from 5 μM to 50 μM, which corresponds to typical concentrations used in published developmental studies on embryonic stem cells (25 μM; Burdon et al., 1999), MDCK cells (30 μM; Khwaja et al 1998), salivary glands (50 μM) (Kashimata et al, 2000) and starfish oocytes (100 μM) (Stephano and Gould, 2000).

At the time of their isolation (E11.5), the kidney rudiments used in this study had developed T-shaped ureteric buds. After 72 hours of culture in normal medium, the buds of these kidneys branched several more times in an organotypic manner (Fig. 3A), as has been described many times elsewhere (Groebstein 1953; Davies and Davey, 1999). When cultured in media supplemented with 10 μM-45 μM PD98059, the balance of elongation and branching of the ureteric buds was greatly disturbed so that they produced long tubules with few branches.
mainly at the tips of the ureteric bud, which show the swollen appearance typical of the termini of branching epithelial tubules (Fig. 4A). This localisation of proliferation to the ureteric bud tips has not been described before. Ureteric buds grown in the presence of 25 µM PD98059 also show some proliferation at the bud tips, but it is reduced compared with controls (Fig. 4B). We compared proliferation quantitatively by counting the total number of BrdU-incorporating nuclei within a segment of each tubule between its tip and a point approximately 110 µm (precisely 3.00 arbitrary units on our image analysis system) proximal to it. Data from more than 20 ureteric bud tips were pooled for statistical analysis, which revealed a mean of 15.6 BrdU-positive nuclei in controls, but only 6.75 in those treated with PD98059; a t-test (assuming unequal variances) demonstrated that this was a highly significant difference (P=5.2x10^{-5}). The lower but non-zero rate of cell proliferation in PD98059-treated ureteric buds accords with the observation that some extension continues (Fig. 3). There was very little apoptosis (0.5-1% of nuclei condensed) in the ureteric buds of either control or PD-98059-treated kidneys (Fig. 4C,D), and there was no significant difference between control and PD98059-treated samples. The rarity of apoptotic cells in ureteric buds agrees with the results of Coles et al. (Coles et al., 1993), who used a similar propidium iodide technique to examine cell death in embryonic rat kidneys. Apoptotic figures could, however, be seen in the mesenchymes, as has been described elsewhere (Coles et al., 1993); again, this appeared in both control and experimental samples.

Little is known about the role of the cytoskeleton in ureteric bud branching, but actin/myosin contraction is known to be important in several examples of epithelial morphogenesis, including wound healing, folding of the colon and branching of the salivary gland (Nakanishi and Ishii, 1989; Coloni and Conforti, 1993; McCluskey and Martin, 1995). We have therefore compared the distribution of filamentous actin in normal kidneys and those treated with PD98059. In normal kidneys, there is an intense concentration of actin filaments in the apices of cells at the tips of the ureteric bud (Fig. 4E), but in PD98059 treated kidneys this effect is lost and the concentration of actin filaments at the tip is similar to that in the rest of the ureteric bud (Fig. 4F). One other marker of ureteric buds tips is the presence at the tip of fine processes of laminin that appear to extend into the mesenchyme from the basement membrane (Davies et al., 1995). These can be seen pointing in several directions, with respect to the ureteric bud, from the tips of ureteric buds grown in control medium (Fig. 4A). By contrast, ureteric buds of kidneys grown in the presence of PD98059 showed very few laminin processes, and those that existed were typically found facing only ‘forwards’ with respect to the bud, as shown in Fig. 4B.

Cell proliferation, apoptosis and cytoskeletal arrangement are aspects of cell biology that are likely to be closely involved with the actual production of morphological change, ‘downstream’ of signals that induce that change. Other markers of ureteric bud exist that are likely to be ‘upstream’, for example, the expression of the receptor tyrosine kinases Ret, the receptor for the GDNF family (Schuchardt et al., 1994; Sainio et al., 1997) and Ros, whose ligand is unknown (Tessarollo et al., 1992). Immunofluorescent detection showed that Ret was expressed by the ureteric buds of both control and 25 µM PD98059-treated kidneys. In both cases, expression

Fig. 3. Blocking the Erk MAP kinase pathway using PD98059 inhibits ureteric bud branching. (A) The ureteric buds of E11.5 kidney rudiments cultured for 72 hours in normal medium branch extensively in an organotypic manner. (B,C) Inclusion of PD98059 (15 µM), an inhibitor of Mek-activation of Erk1/2 MAP kinases, greatly decreases the number of branches produced by the ureteric bud although some elongation of existing branches continues. (D) When kidney rudiments are grown for 40 hours in PD98059 (10 µM) and then transferred to normal medium for a further 48 hours, the central, oldest parts of the ureteric bud tree show sparse branching but the outer (newest) parts show a more normal branching density, suggesting that the cells recover from PD98059 treatment. (E) A dose-response curve for PD98059, showing how the number of tips formed by the ureteric bud tree declines with increasing (PD98059); error bars represent the standard error of the mean.

(Fig. 3B,C). Lower concentrations of PD98059 reduced but did not eliminate branching (see Fig. 3E for a dose-response curve). The effect of PD98059 was reversible, so that kidney rudiments cultured in the presence of PD98059 for 40 hours and then placed for a further 48 hours in standard medium produced an arbour, the medullary (older) part of which had long under-branched tubules and the cortical (newer) parts had an apparently normal density of new branches (Fig. 3D). Western blotting for phosphorylated Erk1/2 confirmed that 20 µM PD98059 almost eliminated Erk activation without affecting the amounts of Erk protein present (see Fig. 7, rightmost track).

To understand more about the means by which the Erk1/2 pathway controls growth of the ureteric bud, we examined proliferation and apoptosis of bud cells using BrdU incorporation and nuclear morphology. Cell proliferation in the ureteric buds of kidneys cultured in normal medium is located
Erk in developing kidney could be detected throughout the bud (Fig. 4G,H). This pattern is normal for such young, small buds, and has been described before at both the mRNA and protein levels (Pachnis et al., 1993; Liu et al., 1996); only later in embryonic development, when the bud is larger, is Ret lost from the bud stalk. Immunofluorescent detection of Ros again showed the molecule to be present in the presence or absence of 25 μM PD98059 (Fig. 4I,J). It was present throughout the bud, which is again normal for these early stages of development and has been described before (Kanwar et al., 1995). A subtle difference could, however, be detected using high magnification confocal microscopy; in about half of the ureteric bud tips of normal kidneys, expression of Ros in the basal surface of the cells, where it is generally strong, is reduced in the few cells right at the tip (Fig. 4J, arrow), but this effect was never seen in kidneys incubated in PD98059.

In summary, the overall effect of inhibiting the activation of Erk1/2 was to inhibit ureteric bud branching while allowing some extension. At a cellular level, rates of apoptosis in the bud showed no detectable change but rates of cell proliferation fell significantly, the concentration of actin filaments typical of branching tips was lost and there was little production of laminin processes typical of normal bud tips. Expression of Ret and Ros receptor tyrosine kinases was, however, maintained at least for the duration of these experiments (1-2 days).

**An active Erk MAP kinase pathway is required by the ureteric bud itself**

Active Erk MAP-kinase is expressed by both the ureteric bud and the mesenchyme that surrounds it. The inhibition of ureteric bud development caused by inhibition of the MAP kinase pathway might therefore be explained by an indirect mechanism in which the mesenchyme requires active MAP kinase in order to produce an environment that will support bud arborisation. To discriminate between this possibility and a direct requirement for Erk activation in the bud itself, it was necessary to examine the behaviour of the ureteric bud, with and without MAP kinase inhibitors, in a host tissue other than metanephrogenic mesenchyme. Most other mesenchymes will not support ureteric bud development, but lung mesenchyme will do so in media supplemented with exogenous GDNF (Sainio et al., 1997). We have found that branching morphogenesis of E11 murine lung is relatively unaffected by concentrations of PD98059 (up to 25 μM) that inhibit branching in the kidney, and even at 75 μM PD98059, some lung branching continues (Fig. 5). Lung mesenchyme is therefore still able to support epithelial arborisation even in 25 μM PD98059.

The necessity for Erks in the ureteric bud was tested by combining isolated ureteric bud with lung mesenchymes in the presence or absence of PD98059. In the absence of PD98059, the ureteric bud arborised in the lung mesenchyme and showed an obviously renal-type branch pattern. In the presence of 25 μM PD98059, however, the ureteric bud showed the same inhibition of branching in lung mesenchyme as it does in kidney mesenchyme (Fig. 5F). As lung mesenchyme is able to support branching of its own epithelium under these conditions, the ureteric bud itself must require active Erk MAP kinases for branching morphogenesis to take place.

The hypothesis that MAP kinase activity is required by the ureteric bud itself was further tested by exploiting the hanging

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**Fig. 4.** Effect of PD98059 on ureteric bud cell biology. (A) The pattern of cell proliferation (BrdU incorporation) in normal kidneys; the inset shows both ureteric bud and mesenchyme, and the main image shows only the ureteric bud for clarity (the mesenchyme being masked out electronically). Most BrdU incorporation (green) takes place in the ureteric bud tips. The laminin basement membrane of those tips (red) shows numerous short processes reaching out into the mesenchyme (arrowheads). (B) In kidneys cultures in the presence of 25 μM PD98059, there is less proliferation in the ureteric bud, though it is again localised mainly in the tips. There are also fewer processes and typically, as in this image, the only process points directly forward from the tip (arrowhead). (C,D) Propidium iodide staining of nuclei reveals very little apoptosis in the ureteric buds of either PD98059-treated (C) or control (D) kidneys, although in both cases there is mesenchymal apoptosis away from the immediate vicinity of the ureteric bud (arrowheads). (E,F) Filamentous actin is concentrated strongly at the apices of cells of control kidneys (E) (arrowheads), but much less so in kidneys growing in PD98059 (F). (G,H) Ureteric buds of both PD98059-treated kidneys (G) and controls (H) express Ret. (I,J) Ureteric buds of both PD98059-treated (I) and control (J) kidneys express Ros; many tips of control kidneys show reduced immunoreactivity in the few cells right at the ends of the tips (arrowhead), though PD98059-treated kidneys do not show this effect. Scale bars: 50 μm.
drop culture system of Sainio et al. (Sainio et al., 1997). These authors showed that ureteric buds cultured in hanging drops would lose their integrity over the course of 24 hours or so, their cells parting company to form a diffuse mass with a mesenchyme-like appearance. Inclusion of GDNF in the culture medium, however, maintained the epithelial appearance of the buds so that they did not break up. The maintenance of epithelial morphology in hanging drops when provided with adequate growth factors is one observable aspect of ureteric bud behaviour that does not require the influence of a supporting mesenchyme. We have therefore tested the effect of MAP-kinase inhibition on ureteric bud behaviour in this mesenchyme-free system. 50 ng/ml GDNF maintained the epithelial morphology of isolated ureteric buds (Fig. 5G), as described by Sainio et al. (Sainio et al., 1997), but inclusion of 10-20 μM PD98059 in the culture medium abrogated this effect, allowing the ureteric buds to shed cells and to lose their integrity as if no GDNF were present (H).

Erk phosphorylation is regulated by GDNF signalling and by sulphated glycosaminoglycans, though not detectably by BMP2

If the MAP kinase pathway is a genuine regulator intracellular of ureteric bud branching, it would be expected to be affected by at least one extracellular regulator of branching. Many extracellular regulators probably remain to be identified, but several are known, including components of the cell matrix (laminins, nidogen and sulphated glycosaminoglycans) (Ekblom et al., 1994; Davies et al., 1995; Kispert et al., 1996; Bullock et al., 1998) and growth factors (GDNF, neurturin, persephin, HGF, BMP2, BMP7, TGFβ) (Woolf et al., 1995; Ritvos et al., 1995; Vega et al., 1996; Vukicevic et al., 1996; Sainio et al., 1997; Milbrandt et al., 1998; Davies et al., 1999; Gupta et al., 1999). Of these, sulphated glycosaminoglycans and GDNF are outstanding because their importance has been demonstrated by both transgenic and culture studies (Pichel et al., 1996; Moore et al., 1996; Davies et al., 1995; Bullock et al., 1998). We therefore investigated whether either of these two extracellular regulators of ureteric bud branching play a role in controlling the intracellular MAP kinase pathway.

The connection between GDNF signalling and Erk MAP kinases was explored by using anti-GDNF antibodies, which have already been shown to inhibit ureteric bud branching strongly (Vega et al., 1996; Davies et al., 1999). As expected, kidney rudiments incubated in 10 μg/ml anti-GDNF showed a marked reduction of ureteric bud development after 48 hours compared with controls (Fig. 6). Analysis of Erk phosphorylation in ureteric buds after this length of incubation was not possible, because the anatomy of control kidneys had become too complex for the ureteric bud to be isolated, but it was possible to isolate ureteric buds, by dissection in cold medium, from both experimental and control antibodies after a 7 hour incubation with and without 10 μg/ml anti-GDNF.

Western blotting of these samples showed that anti-GDNF treatment reduced phosphorylation of Erk MAP kinase without affecting the net amount of Erk protein in the tissues (Fig. 6). GDNF is therefore a significant regulator of Erk activation in the developing ureteric bud.

To establish whether the presence of sulphated glycosaminoglycans in the kidney is important to Erk activation, their synthesis was prevented by addition of 30 mM sodium chlorate to the culture medium. The use of chlorate, which blocks sulphation of glycosaminoglycans during their synthesis by competing with sulphate ions for sulphotransferase enzymes, is a standard and well-characterised method that we have described before; it results
in cessation of ureteric bud arborisation although nephron formation continues (Davies et al., 1995; Milbrandt et al., 1998; Davies et al., 1999). After approximately 72 hours in these culture conditions, levels of Erk MAP kinase activation drop markedly compared with those in control kidneys, suggesting that the MAP kinase pathway in the developing kidney is regulated (however indirectly) by activities of sulphated glycosaminoglycans. This drop in MAP kinase activation is apparent when ureteric buds isolated from kidneys (after 12-13 hour incubation) or when complete kidneys are analysed (Fig. 7).

**DISCUSSION**

We have shown that Erk MAP kinases are normally active in branching ureteric bud, that branching morphogenesis requires their activity and that their inhibition reduces cell proliferation and alters matrix organisation. We have also shown that Erk activation in the ureteric bud requires both GDNF and sulphated glycosaminoglycans.

Signal transduction through the Erk pathway is known to be involved in regulating the branching morphogenesis of epithelium in two other systems, mammalian salivary glands and *Drosophila* tracheae (Kashimata et al., 2000; Gabay et al., 1997). That it should also be involved in development of the ureteric bud raises the possibility that branching morphogenesis may use conserved intracellular mechanisms across a wide variety of systems. There are, however, detailed differences between the three model systems that make comparisons complicated. In both kidney and salivary gland, for example, branching morphogenesis is accompanied by cell
Fig. 8. The activation of Erk MAP kinases in the ureteric bud is not detectably affected when kidneys are incubated in the presence of 10 ng/ml BMP2. (A) The top panel shows a western blot of ureteric buds, isolated after an 8 hour incubation of complete kidneys with and without BMP2, probed with an antibody specific for phosphorylated Erk1/2 (Erk-P). The bottom panel shows the same samples probed with an antibody that recognises both phosphorylated and non-phosphorylated Erk. (B,C) Micrographs of kidneys cultured in normal medium (C) and 10 ng/ml BMP2 (C) for 30 hours confirm that BMP2 inhibits ureteric bud morphogenesis.

Proliferation, whereas the tracheal system of Drosophila develops by cell enlargement and rearrangement without multiplication. In addition, inhibition of Erk activation in salivary glands results in cessation of both growth and extension (Kashimato et al., 2000), but in our experiments with kidneys, tubule extension continued even when branching was inhibited. An involvement of Erks in branching systems with these differences may either indicate that the pathway is truly fundamental and that its importance lies deeper than detailed differences, or it may simply result from Erk being involved in quite different processes in the three organs.

To the best of our knowledge, this is the first report of GDNF signalling being transduced via the Erk MAP kinase pathway in epithelial cells, but a link between GDNF and Erk has been well established in several neuronal cell types. Ret activation, by GDNF and related ligands, activates both Erk MAP kinase and phosphatidylinositol-3-kinase in sympathetic neurons of the superior cervical ganglion, in chick motoneurones and in a motoneuron-derived cell line (Kotzbauer et al., 1996; Creedon et al., 1996; Soler et al., 1999; Trupp, 1999). In chicken motoneurones in primary culture, only the phosphatidylinositol-3-kinase pathway is required for GDNF-mediated cell survival (Soler et al., 1999), suggesting that the function of MAP kinase (if any) may be more to do with neurite outgrowth. Certainly, Erk MAP kinase activity is associated with neurite outgrowth in response to other growth factors in neuronal models such as PC12 cells (Qiu and Green 1992; Cowley et al., 1994; Pang et al., 1995; Fukuda et al., 1995; Creedon et al., 1996). Neurite production usually involves branching as well as growth, particularly in the case of dendritic trees, so the involvement of the Erk pathway in neurite morphogenesis again raises a possibility of a conserved role for the pathway in branching morphogenesis even across different types of tissue.

The dependence of the MAP kinase pathway in the ureteric bud on sulphated glycosaminoglycans is intriguing. Glycosaminoglycans have many known roles, including presentation of growth factors to their high-affinity receptors and organisation of the extracellular matrix. Either role could involve them in regulating epithelial Erk activity. If the Erk MAP kinase cascade in ureteric bud is controlled by the receptor for any growth factors that require presentation by sulphated glycosaminoglycans, then loss of the glycosaminoglycans would produce defective signalling and hence defective activation of MAP kinase. At least two molecules present in developing kidney, FGF2 and HGF, are known to be presented by sulphated glycosaminoglycans (Rapreager et al., 1991; Lyon et al., 1998), and there could be many more, possibly including GDNF itself. Cells sense their matrix via integrin and other matrix receptors, and integrin-containing complexes of cells can signal, via focal adhesion kinase and Grb2, to the Ras-MAP kinase pathway (Chen et al., 1998). Defective matrix organisation caused by lack of sulphated glycosaminoglycans could therefore affect MAP kinase without any link to growth factors. It is difficult to use studies from other cell types for guidance about which is the most likely role of glycosaminoglycans in regulating Erk, because there is such variation in the way that the Erk pathway is controlled. In PC12 pheochromocytoma cells, for example, G-protein-coupled receptor-mediated Erk activation is almost exclusively dependent on focal adhesion kinase, whereas in rat1 fibroblasts it is almost exclusively receptor tyrosine kinases (Della Rocca et al., 1989).

Erks have many effects in different cell types, and all tissues examined so far contain immunoreactive Erk1 and Erk2 (Cobb et al., 1994). Erk was first characterised in the context of proliferative responses of cells to mitogens (Rossomando et al., 1989), and ensuring adequate levels of proliferation may be one important role for Erks in ureteric bud morphogenesis. We found that cell proliferation is concentrated in the very tips of normal ureteric buds, suggesting that elongation, as well as branching, takes place mainly terminally. When Erk activation was prevented, there was significantly less cell proliferation but what remained was still concentrated at the termini of the bud, and was presumably the cause of the tubule elongation that still took place. It seems unlikely, however, that reduced proliferation can alone account for the absence of branching; given that some elongation continues, something else must account for the fact that the elongation is now directed only forwards. In addition, we have previously reported that direct inhibition of cell cycling, using methotrexate, has opposite effects to those of MAP kinase inhibition, in that branch initiation continues but elongation ceases (Davies et al., 1995).

One other possible function of the Erk pathway is modulation of adhesion between ureteric bud cells. We found the Erk pathway to be necessary for GDNF to maintain isolated ureteric buds as adhering, epithelial cysts and prevent them breaking up into loosely aggregated scattered cells, as happens in the absence of GDNF. This is an interesting result, in that it implies that Erk can modulate cell adhesion in this tissue, but is also paradoxical (as was the original observation of Sainio et al. that GDNF maintains integrity (Sainio et al., 1997), for
in other systems branching morphogenesis is associated with molecules that promote cell scatter (e.g. HGF) (Santos and Nigam, 1993).

The tips of ureteric buds possess fine processes rich in laminin, contiguous with the basement membrane (at least at the resolution of light microscopy), that reach out into the surrounding mesenchyme (Davies et al., 1995). In kidneys, growing in normal media, these processes emanate from several points of the distended tips, so that they point in several directions, but in kidneys treated with MAP kinase kinase inhibitor, there are few processes and they point only forwards. The nature and function of these processes has yet to be elucidated, but in the light of the correlation presented here – that processes project forwards and sideways in branching ureteric buds, but only forwards in ureteric buds that only elongate – it is tempting to speculate that the elongating ureteric bud may follow where they lead. In that case, whatever effect the Erk pathway has on process formation would be a primary effector of branching. The Erk pathway has been shown to interact with the cytoskeleton, which might be a potent effector of branching morphogenesis in ureteric bud as it is known to be in salivary gland (Nakanishi and Ishii, 1989). In particular, Erks interact with both the actin-binding protein, calponin, and with actin itself (Leinweber et al., 1999). This may account for our observations that inhibition of Erks results in a failure of ureteric bud tips to produce their normal pattern of actin filaments. In some cells, at least, the Erk pathway can also interact with other protein kinases in the cytoplasm and on membrane receptors such as EGF-R (Davis, 1995; Tibbles and Woodgett, 1999), so that it could modulate the effects of other regulators of ureteric bud development.

The morphological effect of inhibiting Erk activation in the kidney is unusual, in that most other treatments that inhibit ureteric bud morphogenesis in culture (e.g. BMP2, anti-GDNF, anti-HGF, chlorate, glycanases, etc.) inhibit both branch initiation and duct elongation. Inhibition of Erk activation inhibits branching but at least some elongation continues. Continued elongation in the absence of branching strengthens previous suggestions that growth and branching of the ureteric bud may be controlled separately, the earlier observations being that TGFβ seems to encourage elongation at the expense of branching, and that treatment of rudiments deprived of sulphated glycosaminoglycans with HGF ‘rescues’ elongation without rescuing branching (Ritvos et al., 1995; Davies et al., 1995). Separate control of branching and elongation could in principle allow more flexibility of the shape of a branched epithelial system over both developmental and evolutionary time, and in the particular case of the kidney, this flexibility may be used when the simple, frequently branching dichotomous pattern of early development gives way to the ‘arcade’ production of late development, in which elongation dominates (al-Awqati and Goldberg, 1998).

Three ‘classical’ signal transduction proteins have now been implicated in ureteric bud development: protein kinase C, which induces branching (Davies et al., 1995); protein kinase A, which inhibits branching (Gupta et al., 1999); and Erk MAP kinase, which is necessary for branching (this report). The protein kinase A pathway is regulated by (at least) BMP2 and BMP7, and the Erk MAP kinase pathway by (at least) GDNF and glycosaminoglycans. In order to understand how these paths integrate the many regulatory influences on the ureteric bud, it will be important to determine which other known regulators lie upstream of them, how they regulate each other and, most crucially, to identify the intracellular targets of these pathways that link them to final effectors of morphogenesis.

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