Both mitogen activated protein kinase and the mammalian target of rapamycin modulate the development of functional renal proximal tubules in matrigel

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Summary

Tubules may arise during branching morphogenesis through several mechanisms including wrapping, budding, cavitation and cord hollowing. In this report we present evidence that is consistent with renal proximal tubule formation through a process of cord hollowing (a process that requires the concomitant establishment of apicobasal polarity and lumen formation). Pockets of lumen filled with Lucifer Yellow were observed within developing cords of rabbit renal proximal tubule cells in matrigel. The observation of Lucifer Yellow accumulation suggests functional polarization. In the renal proximal tubule Lucifer Yellow is initially transported intracellularly by means of a basolaterally oriented p-aminohippurate transport system, followed by apical secretion into the lumen of the nephron. Consistent with such polarization in developing tubules, *Triticum vulgare* was observed to bind to the lumenal membranes within pockets of Lucifer Yellow-filled lumens. As this lectin binds apically in the rabbit renal proximal tubule, *T. vulgare* binding is indicative of the emergence of an apical domain before the formation of a contiguous lumen. Both epidermal growth factor and hepatocyte growth factor stimulated the formation of transporting tubules. The stimulatory effect of both epidermal growth factor and hepatocyte growth factor on tubulogenesis was inhibited by PD98059, a mitogen activated protein kinase kinase inhibitor, rather than by wortmannin, an inhibitor of phosphoinositide 3-kinase. Nevertheless, Lucifer Yellow-filled lumens were observed in tubules that formed in the presence of PD98059 as well as with wortmannin, indicating that these drugs did not prevent the process of cavitation. By contrast, rapamycin, an inhibitor of the mammalian target of rapamycin, prevented the process of cavitation without affecting the frequency of formation of developing cords. Multicellular cysts were observed to form in 8-bromocyclic AMP-treated cultures. As these cysts did not similarly accumulate Lucifer Yellow lumenally, it is very likely that processes other than organic anion accumulation are involved in the process of cystogenesis, including the Na,K-ATPase.

Key words: Primary culture, Kidney, Tubulogenesis, Matrigel, Transport

Introduction

Epithelial branching morphogenesis has been observed to occur in vitro, in reconstituted basement membranes, as well as in vivo during early development (Grobstein, 1967; Taub et al., 1990; Unsworth and Grobstein, 1970). Growth factors as well as basement membrane proteins have been implicated in playing a role in this process (Klein et al., 1988). Indeed, previously we showed that either epidermal growth factor (EGF), or transforming growth factor α (TGF-α), is required for baby mouse kidney epithelial cells to form tubules in matrigel (Taub et al., 1990). Matrigel is an extract of the EHS tumor, which reconstitutes into a gel-like structure under physiological conditions in vitro (Kleinman et al., 1982). Ultrastructure studies indicate that matrigel is composed of a network of matrix proteins similar to the lamina densa in authentic basement membranes. Indeed, the same proteins present in authentic basement membranes, which include laminin, collagen IV, heparan sulfate proteoglycan and nidogen/entactin, are present in matrigel (Kleinman et al., 1982). The presence of laminin in particular, as well as collagen IV in matrigel, provides this preparation the ability to promote tube formation by human umbilical cord endothelial cells in vitro (Grant et al., 1989).

Several cell types have been reported to form differentiated structures in matrigel. For example, cultured mammary epithelial cells in matrigel may form branched tubules in response to hepatocte growth factor (HGF), as well as alveoli (Niemann et al., 1998). Although human mammary cells in matrigel have been reported to form acini with a hollow, lumenal space (Debnath et al., 2002), mouse testicular tissue

has been reported to simply form cords in matrigel (Hadley et al., 1990). However, the mechanisms underlying the formation of tubules and or acini with lumenal spaces are still unclear. Other than the reports of lumen formation, evidence is lacking as to whether any of these tubules and/or acini that form in vitro are actually functional.

When considering the functions of renal tubules in particular, a central aspect is their capacity for the reabsorption as well as the secretion of solutes. However, studies concerning such renal functions are complicated by differences in the transport properties of tubule epithelial cells in different segments of the nephron. In addition, renal tubules are derived from two distinct embryological structures, the metanephric mesenchyme and the ureteric bud (Stuart and Nigam, 1995). Previously, we reported that the branching tubules formed by primary baby mouse kidney cells in matrigel had a morphology distinctive of the renal collecting duct, which originates from the ureteric bud (Taub et al., 1990). This report is concerned with determining whether renal tubulogenesis in matrigel occurs by means of either cavitation, cord hollowing or wrapping (Lubarsky and Krasnow, 2003). The signaling pathways by which growth factors such as EGF stimulate this morphological process are also studied.

Primary rabbit kidney proximal tubule (RPT) cell cultures are particularly appropriate for such studies. The renal proximal tubule is of mesenchymal origin, permitting us to assess whether such cells are capable of forming tubules in matrigel, as previously observed with cells originating from the ureteric bud. The primary RPT cells retain several differentiated functions when maintained as monolayer cultures in hormonally defined serum-free medium (Chung et al., 1982). In addition to retaining a polarized morphology (Taub et al., 1998), the primary RPT cells possess brush border enzymes (including alkaline phosphatase and γ-glutamyltranspeptidase) and gluconeogenic capacity (Jung et al., 1992; Wang and Taub, 1991), as well as hormone responses typical of the renal proximal tubule (Chung et al., 1982; Han et al., 1999; Han et al., 2000; Wang and Taub, 1991), including the parathyroid hormone-sensitive cyclic AMP production. Evidence has been obtained for the presence of distinctive renal proximal tubule transport systems involved in reabsorption, including an apical Na^+/glucose cotransport system (Chung et al., 1982; Taub et al., 1998), as well as transport systems involved in secretion, including the basolateral p-aminohippurate (PAH) transport (Yang et al., 1988).

In this report primary RPT cells in matrigel are used to assess the mechanisms by which kidney proximal tubules develop to form lumens, establish functional polarity and develop the capacity for transepithelial solute transport. We find that primary rabbit kidney cells do indeed possess the capacity to form proximal tubules in matrigel, which accumulate Lucifer Yellow (a substrate of the PAH transport system) into the lumen of the tubules. Thus, the emergence of transepithelial transport is assessed through the accumulation of Lucifer Yellow in the lumen, while the development of the apical domain is evaluated using lectins. Evidence is presented indicating that tubules are produced by a process of cord hollowing. In experiments using specific inhibitors, evidence is presented indicating that the formation of cords involves the mitogen activated protein (MAP) kinase signaling pathway, while the process of cord hollowing involves the mammalian target of rapamycin (mTOR). The involvement of cAMP in the formation of renal cysts in matrigel is also studied.

Materials and Methods

Culture medium

The basal medium consisted of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F12 medium (F12) (pH 7.4) containing 15 mM HEPES, 20 mM sodium bicarbonate, penicillin (92 units/ml) and kanamycin (0.1%) (DME/F12). Immediately before use, the medium was further supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), 50 nM hydrocortisone, and, if appropriate, other factors, including epidermal growth factor (EGF) (10 ng/ml) (Chung et al., 1982). In experiments where Lucifer Yellow was used, DME/F12 lacking Phenol Red, penicillin and kanamycin (Phenol Red-free DME/F12) was employed. Water used for medium and growth factor preparation was purified using a Milli-Q deionization system.

Primary rabbit kidney proximal tubule cell culture

Primary rabbit kidney proximal tubule (RPT) cell cultures were prepared by a modification of the method previously described (Chung et al., 1982). To summarize, a kidney obtained from a male New Zealand White rabbit (2-2.5 kg) was perfused via the renal artery, first with phosphate buffered saline (PBS), and subsequently with 0.5% (w/v) iron oxide in PBS. The renal cortex was sliced, homogenized with a sterile Dounce homogenizer (loose pestle), and the homogenate (consisting of tubule segments) was sequentially passed through a 253 μm and an 83 μm mesh. The renal proximal tubules and the glomeruli on the 83 μm mesh were transferred into a 50 ml conical tube containing DME/F12 medium. Glomeruli (with iron oxide) were then removed with a sterile stir bar. The remaining tubules were washed twice by centrifugation and resuspended in matrigel. The iron oxide utilized in these studies was obtained from stock solutions in 0.9% NaCl, which were sterilized in an autoclave, and diluted with PBS before use (Cook and Pickering, 1958).

Matrigel cultures

Matrigel was prepared from an unfractionated high-salt/urea extract of the EHS tumor (Kleinman et al., 1986), and stored at –20°C. Growth factor depleted matrigel was obtained from normal matrigel by precipitation with 20% ammonium sulfate (Taub et al., 1990). Before it was used, matrigel was thawed and maintained at 4°C until the renal material was added. Then, the matrigel containing renal material was transferred into 35 mm culture dishes precoated with matrigel. Matrigel cultures were maintained in a humidified 5% CO_2/95% air environment at 37°C. Medium containing insulin, transferrin, hydrocortisone and other pertinent factors (such as EGF) was added the day after plating.

When quantitating the number of tubules (and/or cysts), matrigel cultures were incubated with either EGF, HGF or other appropriate supplements. One week later, the number of tubules (or cysts) was determined in each of 25 microscope fields. Means±standard errors (s.e.m.) were obtained from determinations made in three dishes for each condition, and were compared with control values in the absence of added growth factor. The difference between two different mean values was analyzed by an analysis of variance (ANOVA). The difference was considered statistically significant when $P<0.05$.

Confocal microscopy

Matrigel cultures were initiated as described above. After 1 week in culture, when tubule formation was observed under an inverted microscope, the cultures were used in confocal microscopy studies.

Twenty hours before their use for confocal microscopy, the medium of the matrigel cultures was changed to Phenol Red-free DME/F12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone and 10 ng/ml EGF. Lucifer Yellow (80 μM), DiI (1 μM) and/or other fluorescent conjugates (e.g. FITC, TRITC or Texas Red) were also added to the culture medium at this time. After 20 hours (and immediately before microscopy) the matrigel cultures were washed six times with PBS and mounted on the stage of a Nikon Optiphot-2 inverted microscope. Confocal images of the cultures were acquired using a BioRad 1024 system mounted on a Nikon Optiphot-2 with a 25X Zeiss Plan-Neofluor 0.8 NA objective. Collection filters used were: 522±16 nm (Lucifer Yellow) and 598±20 nm (DiI). Images were collected at 10% laser power. Confocal images were viewed on a high resolution 21 inch monitor, and saved onto a hard drive. Lucifer Yellow presumably entered the renal proximal tubule cells present in the tubules in matrigel by the basolateral PAH transport system, and was transported out of the cells' apical membrane, diffusing throughout the lumenal space. Similarly, Bodipy Fl verapamil and Rhodamine 123 presumably entered the cells through a basolateral transport system. Rhodamine 123 was then presumably transported out of the cells by the apical multidrug resistant transport system, to diffuse throughout the lumen. Particular lectins also apparently entered the renal proximal tubule cells in matrigel, presumably by endocytosis. TRITC *Triticum vulgare* (wheat germ agglutinin) apparently gained access to the apical domain by a process of transcytosis, and this possibility was examined experimentally by incubating matrigel cultures with both brefeldin A and *T. vulgare* before confocal microscopy.

Light and transmission electron microscopy

Matrigel cultures in 35 mm dishes were examined by means of an inverted microscope, and photographed at 100× magnification. In addition to microscopy of viable cultures, paraffin sections of matrigel cultures were examined. For this purpose, the matrigel cultures were fixed in 10% phosphate buffered formalin, processed for light microscopy by conventional procedures, embedded in paraffin and sectioned at 4 μm. Sections were stained with hematoxylin and eosin.

Primary RPT cells in matrigel to be used for transmission electron microscopy (TEM) were plated on a glass coverslip (22×22 mm) in a 35 mm dish. DME/F12 supplemented with insulin, transferrin, hydrocortisone and EGF was added the day after plating. After 10 days, the cultures were fixed with glutaraldehyde (2% (vol/vol)) in 0.05 M sodium cacodylate (pH 7.4)), and postfixed, first with 0.5% osmium tetroxide/0.8% $K_3Fe(CN)6$/0.05 M cacodylate (pH 7.4), then with 0.15% tannic acid/0.05 M cacodylate (pH 7.4) and finally with 2% (wt/vol) uranyl acetate. The cultures were subsequently dehydrated in acetone, and placed in a 21 mm^3 histomold (Peel-A-Way-Products) containing Eponaraldite (Electron-Microscopy Sciences). Both semi-thin (0.5 μm) and thin (90 nm) sections were cut at 90° relative to the plane of the coverslip, and photographed with a JEOL 100CXII electron microscope.

Northern analysis

RNA was purified using guanidinium isothiocyanate followed by cesium chloride centrifugation (Chirgwin et al., 1979). The purified RNA (10 μg/sample) was separated by electrophoresis in formaldehyde gels and transferred to Zeta Probe Blotting Membranes. The quantity and quality of the RNA was verified by staining duplicate samples with ethidium bromide. Restriction fragments containing cDNA to the dog Na,K-ATPase α1 and β1 subunits (Brown et al., 1987), chicken mitochondrial phosphoenol pyruvate carboxykinase (mPEPCK) (Weldon et al., 1990), and chicken β-actin (Cleveland et al., 1980) were utilized to synthesize [^{32}P]-labeled probes by the random primer method, using [α-^{32}P]CTP. The northern blots were hybridized with the [^{32}P]-labeled probes, and washed (Church and Gilbert, 1984). Subsequently, the radioactive bands on the blots were detected using Kodak XAR X-Ray film. To compare the intensity of the bands, the X-Ray film was first scanned with a Biorad Scanner, followed by densitometry using the Quantity One program.

Cell growth studies

Primary RPT cell cultures were initiated in 35 mm dishes, and counted as previously described (Chung et al., 1982). To summarize, cells were removed periodically from representative culture dishes using 0.05% trypsin/0.5 mM EDTA in PBS. Then the cells were resuspended in PBS and counted in a Coulter Model ZF particle counter. Values are averages of triplicate determinations.

Materials

Insulin, transferrin, hydrocortisone, Lucifer Yellow CH and other chemicals were from Sigma Aldrich Chemical Corp. (St Louis, MO). Powdered DME and F12 medium, soybean trypsin inhibitor, EGF and the RNA ladder were from Invitrogen (Carlsbad, CA). Class IV collagenase was from Worthington (Freehold, NJ). [α-^{32}P]CTP (3000 Ci/mmol) was from Perkin Elmer (Wellesley, MA). Zeta Probe Blotting Membrane was from Biorad (Hercules, CA). TRITC-labeled *T. vulgare* was from Sigma-Aldrich. Other lectins, including FITC-labeled *Dolichos biflorous*, FITC-labeled *Tetragonolobus purpurea*, TRITC-labeled glycine max and Texas Red-labeled *T. vulgare* were from Molecular Probes (Eugene, OR). 5,6-Carboxydichlorofluorecein diacetate, BODIPY FL verapamil, Rhodamine 123 and vibrant 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocyanine perchlorate (DiI) were also from Molecular Probes. TGF-β1 and HGF were from R&D Systems (Minneapolis, MN).

Results

Tubule formation over time

Primary rabbit kidney proximal tubule cells were observed to form unbranched (Fig. 1a), as well as branched tubules in matrigel (Fig. 1b). The day after plating, the original nephron segments became structurally rearranged (forming less organized clusters of cells) with tubule-like structures emerging from the clusters of cells (Fig. 2a). By the next day, these 'tubules' emerged even more (Fig. 2b), followed by extensive branching on day 4 (Fig. 2c).

Transepithelial solute transport occurs in renal proximal tubules with either complete or even with partially formed lumens

To determine whether these 'tubules' were indeed functional, the matrigel cultures were incubated with Lucifer Yellow, a fluorescent substrate of the p-aminohippurate (PAH) transport system (Masereeuw et al., 1999). The PAH transport system, distinctive of the renal proximal tubule, is localized to the cells' basolateral membrane. After organic anions enter renal proximal tubule cells via this transport system, these substrates are secreted through the apical membrane into the lumen of the tubule (Russel et al., 2002). We examined the possibility that Lucifer Yellow was transported in this manner in 2-week-old matrigel cultures. Indeed, following the incubation of matrigel cultures with Lucifer Yellow in Phenol Red-free medium, evidence was obtained that Lucifer Yellow had accumulated into the lumenal space of the tubules (Fig. 3A,B), unlike the case with medium containing Phenol Red (also a substrate of

the pAH transport system) (M.T., unpublished). Fig. 3A,B also indicates a relatively uniform diffusion of Lucifer Yellow throughout the lumen. In younger cultures (only 1 week old), Lucifer Yellow accumulation was also observed, although tubules possessed several distinct lumenal spaces, separated by cellular membranes, rather than possessing a single contiguous lumen (Fig. 3C). Presumably, the process of lumen formation was still occurring in these nephron segments, and further maturation of the cells and/or the membranes would result in the formation of tubules with contiguous lumens as observed in Fig. 3C. In addition to these confocal studies, lumen formation by a branching nephron was also observed by means of TEM (Fig. 3D).

To examine the specificity of substrate accumulation by matrigel cultures, the accumulation of several other fluorescent substrates was examined, including 5,6-carboxyfluorescein (5,6-FAM), a substrate of the PAH transport system (Miller et al., 1996), as well as Rhodamine 123 and BODIPY FL verapamil, two substrates of the MDR-1 encoded p-glycoprotein (pgp) transport system. Rhodamine 123 is an organic substrate of pgp-mediated active drug transport, whereas BODIPY FL verapamil is a substrate of pgp-mediated electrodiffusive anion transport capacity (Altenberg et al., 1994; Lelong et al., 1991).

The accumulation of 5,6-FAM into the lumen of a tubule in matrigel is illustrated in Fig. 4A, and a bright-field image of the same tubule is shown in Fig. 4B. BODIPY FL verapamil only accumulated intracellularly (Fig. 4C), whereas Rhodamine 123 was observed to accumulate into the lumen of tubules in matrigel (Fig. 4D).

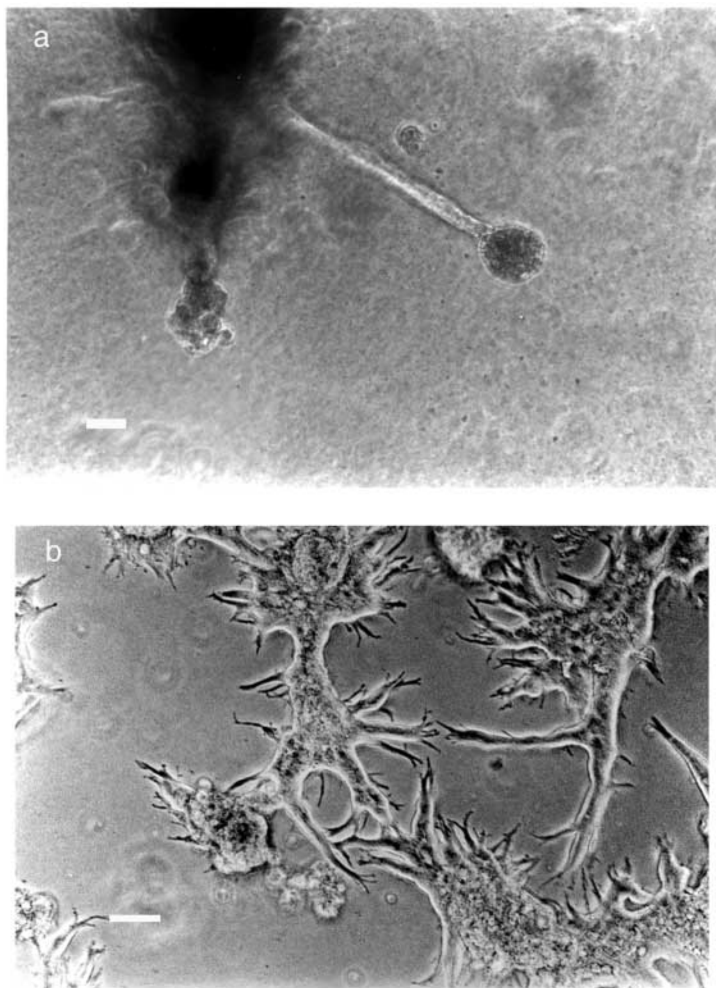

Fig. 1. Tubule formation in response to EGF. Purified rabbit kidney proximal tubules were plated into matrigel in serum free medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone and 10 ng/ml EGF. Subsequently, the cultures were photographed under an inverted microscope. (a) Unbranched tubule, 1 week; (b) branched tubules, 1 week. Bars, 50 μm.

Lumen formation is closely associated with the appearance of an apical membrane as well as the process of transepithelial protein transport

To determine whether the acquisition of the capacity for transepithelial solute transport was associated with the appearance of an apical membrane, developing matrigel cultures were incubated in medium containing the TRITC-labeled lectin *T. vulgare* (or wheat germ agglutinin), which selectively binds to apical membranes in the rabbit renal proximal tubule (Le Hir and Dubach, 1982), in combination with Lucifer Yellow. Fig. 5A shows fluorescent images of a developing tubule overlaid with a bright-field image. *T. vulgare* was observed to bind primarily to membranes facing the developing lumenal spaces, which contain concentrated Lucifer Yellow. The association of the *T. vulgare* lectin with the lumenal membrane presupposes its transport from the antilumenal membrane (facing the matrigel), through the cell to the lumenal surface. Indeed, in matrigel cultures treated with 30 μg/ml brefeldin A, *T. vulgare* was instead primarily localized to the antilumenal membrane (insert, Fig. 5A). Brefeldin A has been reported to inhibit transcytosis presumably by inhibiting protein transport from the endoplasmic reticulum to the Golgi (Low et al., 1991).

The binding of *T. vulgare* to the apical membrane may depend on its recognition of specific binding sites on the apical membrane, in addition to the process of transcytosis. To determine whether the binding specificity of a lectin such as *T. vulgare* could also play a role in determining its localization, the ability of several different lectins to bind to the tubules in matrigel was examined. Both FITC-labeled Lotus *Tetragonolobus purpurea* (winged or asparagus pea lectin) (Fig. 5B) and FITC-labeled *Dolichos biflorous* (horse gram) (Fig. 5C) localized to the cells' antilumenal surface. Once again, the fluorescent images were overlaid with bright-field images of the cells. Fig. 5D shows that, unlike the other lectins studied, TRITC-labeled glycine max (soybean lectin) did not bind extensively to either the lumenal or the antilumenal membrane of transporting tubules (as indicated by the lumenal Lucifer Yellow). Previously, glycine max was observed to bind to the membranes of epithelial cells in all rabbit nephron segments except the renal proximal tubule (Le Hir and Dubach, 1982). Thus, the results of this study indicate that lectin binding specificity in vitro is similar to that observed in intact rabbit renal proximal tubules, and thus that lectin localization in the tubules in matrigel is probably not determined simply by the process of transcytosis.

Formation of functionally polarized tubules in response to HGF as well as EGF

HGF was as effective as EGF in stimulating tubulogenesis (Fig. 6A). However, the tubules that formed in the presence of HGF (Fig. 6B) differed morphologically from the tubules that formed in response to EGF (Fig. 1a,b). Furthermore, HGF has been reported to cause a transient dedifferentiation, and loss of polarity while inducing tubule formation by MDCK cells in a collagen gel (Balkovetz et al., 1997; Pollack et al., 1998). As tubulogenesis by this route may not lead to the formation of a functionally polarized epithelium, we investigated the possibility that the tubules that formed in response to HGF were functional. Indeed, the capacity for these tubules to accumulate Lucifer Yellow intralumenally is indicated by the diffuse fluorescence in the lumen of the tubule shown in Fig. 6C.

Involvement of MAP kinase in the process of tubulogenesis

EGF may stimulate tubulogenesis by means of its affects on cell growth and/or differentiation. Possibly, the stimulatory effect of EGF on tubulogenesis can be explained simply as a consequence of growth stimulation by EGF. To determine whether the stimulatory effects of EGF on growth and tubulogenesis are necessarily interrelated, and whether the stimulatory effects of EGF on tubulogenesis are mediated by either MAP kinase, or phosphoinositide 3-kinase, the effects of PD98059 and wortmannin on tubulogenesis and growth were examined. PD98059 is an inhibitor of MAP kinase kinase (Dudley et al., 1995), while wortmannin is an inhibitor of phosphoinositide 3-kinase (Nakanishi et al., 1992).

EGF caused a significant increase (more than fivefold) in the number of tubules in the matrigel cultures (Fig. 6D). The stimulatory effect of EGF on tubule formation by primary RPT cells was inhibited significantly by PD98059, unlike the case with wortmannin. However, PD98059 and wortmannin did not significantly affect the number of tubules that formed in the absence of EGF. These results indicate that activation of MAP kinase is required in order to elicit the stimulatory effect of EGF on tubule formation, unlike the case with phosphoinositide 3-kinase.

Although the inhibition of tubule formation by PD98059 might be explained by a growth inhibitory effect of this drug, PD98059 alone did not significantly prevent the growth stimulatory effect of EGF in monolayer cultures of primary RPT cells, unless PD98059 was added in combination with wortmannin (Table 1). Thus, although EGF apparently requires MAP kinase activation to stimulate the process of tubulogenesis (a process that is substantially inhibited by PD98059), the stimulatory effect of EGF on tubulogenesis nevertheless cannot simply be attributed to its stimulatory effect on growth (which can continue uninhibited in the presence of PD98059).

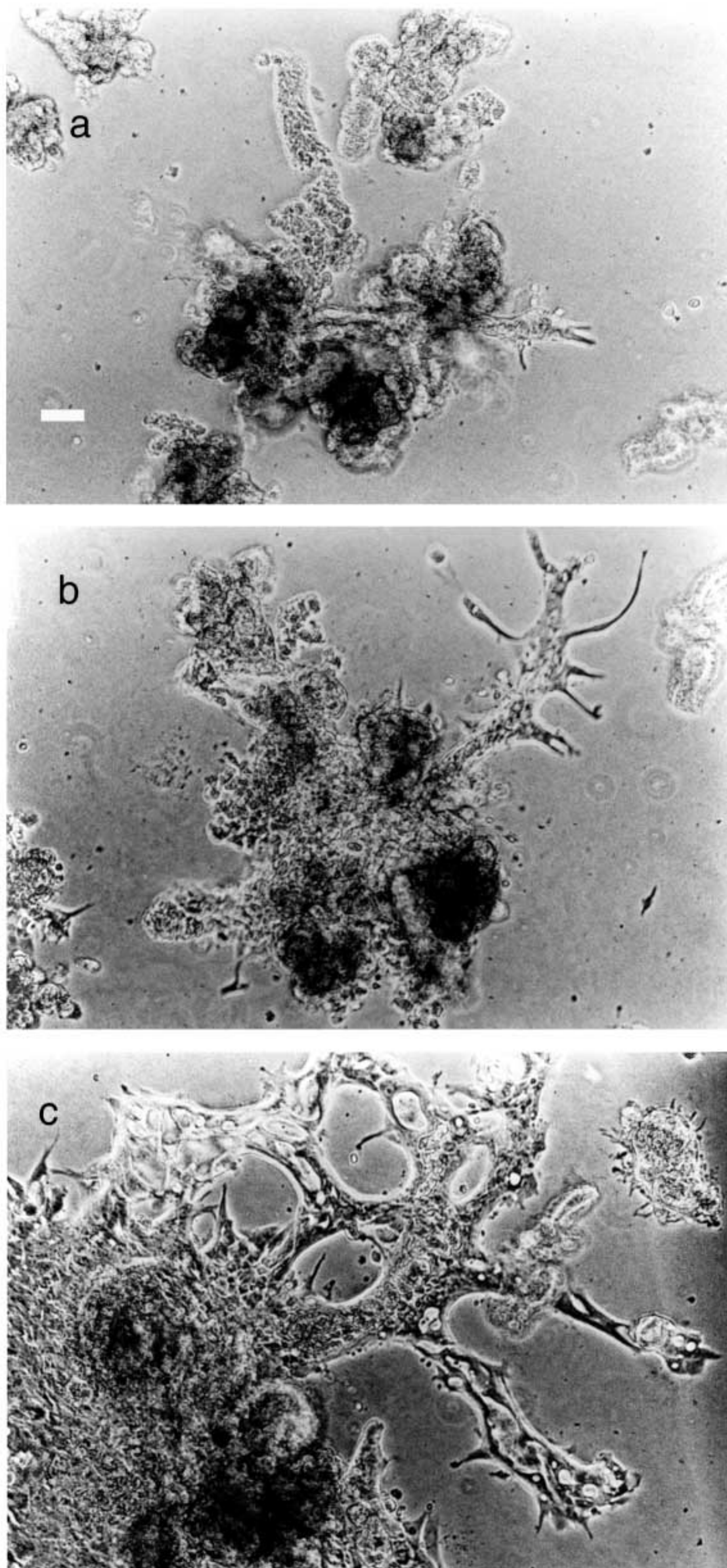

Fig. 2. Tubule formation as a function of time. Matrigel cultures were initiated in serum free medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone and 10 ng/ml EGF, and then examined under an inverted microscope. (a) A microscope field after 1 day in culture. The same microscope field after (b) 2 days and (c) 4 days in culture. Bar in a, 50 μm (same scale for b and c).

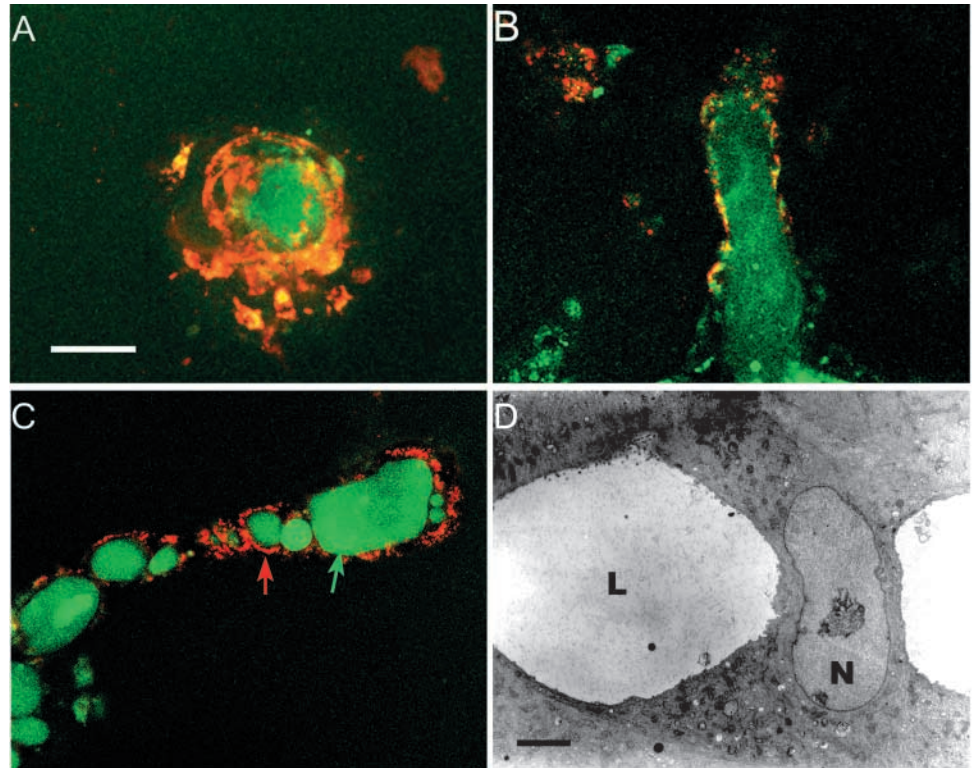

Fig. 3. Lumen formation by tubules. Lucifer Yellow accumulation (observed as green) in the lumen of a tubule in matrigel cultures, while DiI (observed as Yellow) acted as a membrane stain. Matrigel cultures were initiated as described in the legend to Fig. 2. Subsequently, the cultures were incubated for 20 hours in Phenol Red-free medium supplemented with 80 μM Lucifer Yellow, 1 μM DiI, 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5×10^{-8} M hydrocortisone and 10 ng/ml EGF. (A) Transverse section of a 2-week-old culture. (B) Longitudinal section of another tubule. (C) Section of a 1-week-old matrigel culture. Red arrow, DiI stained plasma membrane; Green arrow, Lucifer Yellow in lumen. (D) Cross-section of a tubule formed by a 1-week-old matrigel culture was examined by TEM, showing a lumen (L) and nucleus (N). Bar in A, 50 μm (same scale for B and C); Bar in D, 10 μm.

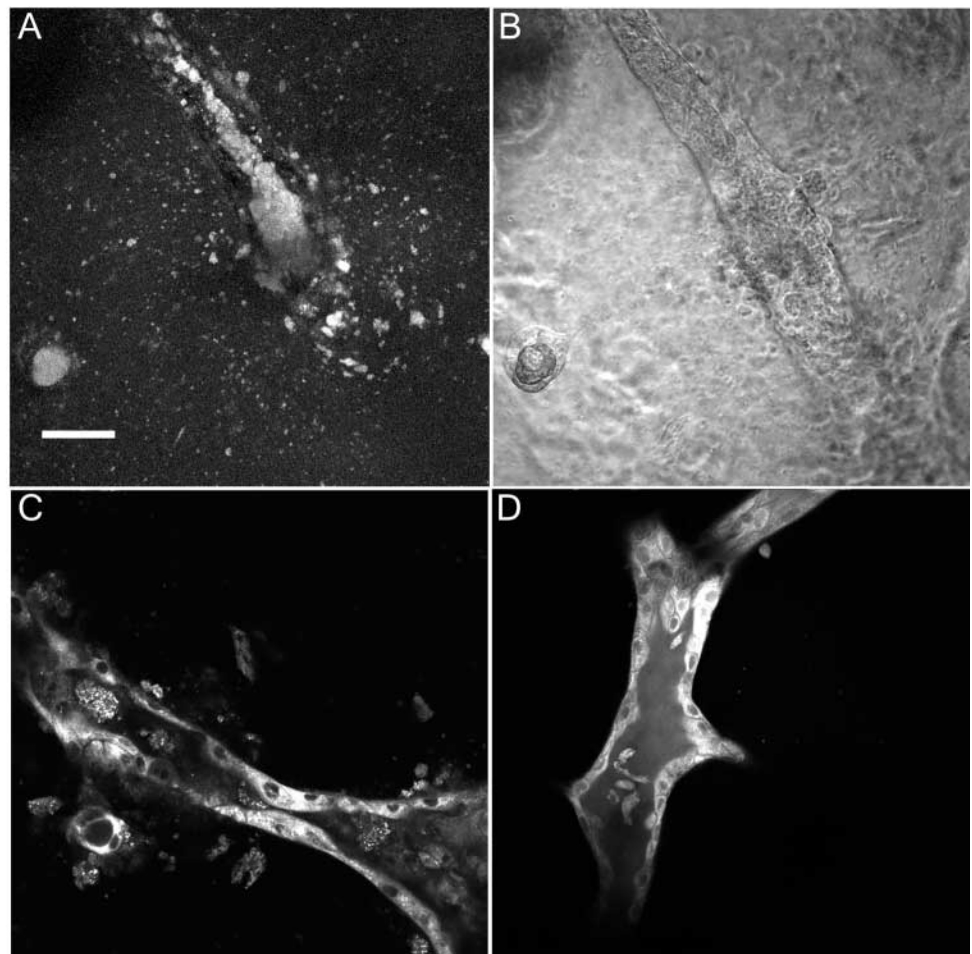

Fig. 4. Specificity of substrate accumulation. To determine whether tubules in matrigel cultures transport a variety of fluorescent substrates, matrigel cultures were initiated as described in the legend to Fig. 2. Confocal micrographs of matrigel cultures incubated for 20 hours in Phenol Red-free medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone, 10 ng/ml EGF and either 5,6-carboxyfluorescein (5 μM), Rhodamine 123 (10 μM) or BODIPY FL verapamil (5 μM), also as described in Fig. 3. (A) Cultures with 5,6-carboxyfluorescein (5,6 FAM) shown as a fluorescent image (white present in the lumen represents 5,6 FAM), and the same section shown in (B) as a bright-field image. (C) Fluorescent image of a culture incubated with BODIPY FL verapamil (represented by white, which is present intracellularly). (D) Fluorescent image of a culture incubated as described above with Rhodamine 123 (represented by white in cells, and diffuse gray in the lumen). Bar in A, 50 μm (same scales for B, C and D).

HGF, like EGF, may have required the activation of MAP kinase to stimulate tubule formation. Indeed, PD98059 was observed to similarly reduce the frequency of tubule formation in the presence of HGF, unlike the case with wortmannin (Fig. 6E). However, unlike the case with EGF, PD98059 was observed to prevent the growth stimulatory effect of HGF (Table 1), indicating that when considering the involvement of p42/p44 MAP kinase in mediating the effect of HGF on tubulogenesis, a significant component of this effect may simply be through increased growth.

Table 1. Effects of regulatory factors on growth

Experiment 1	Control	EGF
Untreated	100±9	185±10
Wortmannin	147±4	229±15
PD 98059	60±4	165±9
Experiment 2	**Control**	**EGF**
Untreated	100±9	251±12
Wortmannin+PD98059	73±3	147±8
Experiment 3	**Control**	**HGF**
Untreated	100±13	203±27
Wortmannin	122±13	190±20
PD 98059	86±17	112±2

Primary RPT cells were grown in the presence of 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5×10^{-8} M hydrocortisone and other appropriate factors, as indicated, including 10 ng/ml EGF, 50 ng/ml HGF, 5×10^{-8} M wortmannin and 5×10^{-5} M PD98059. After 5 days the cultures were counted in triplicate dishes, and the cell number (%) was compared with the control.

Involvement of mTOR in lumen formation

In the studies described above, the effects of PD98059 and wortmannin on the frequency of tubule (or cord) formation were assessed using an inverted microscope. To assess the effects of these two inhibitors on lumen formation, confocal microscope studies were conducted. In addition, the effect of rapamycin, an inhibitor of the mTOR pathway was evaluated. Fig. 7A and Fig. 7B show the lumenal accumulation of Lucifer Yellow into tubules that formed in the presence of either PD98059, or wortmannin, respectively. Although no effect of rapamycin on the frequency of tubule formation was observed, when examining individual tubules under the confocal microscope, we observed (as shown in Fig. 7C) that the so-called tubules that formed in cultures treated with rapamycin actually consisted of a solid cylindrical mass of cells, in which some cavitation had apparently occurred. Indeed, several small pockets of Lucifer Yellow were observed within the cylindrical mass.

Effect of 8-bromocyclic AMP on cyst formation

When 8-bromocyclic AMP (8-Br-cAMP) was added to the matrigel cultures renal cysts formed (Fig. 8a,b), which seemed

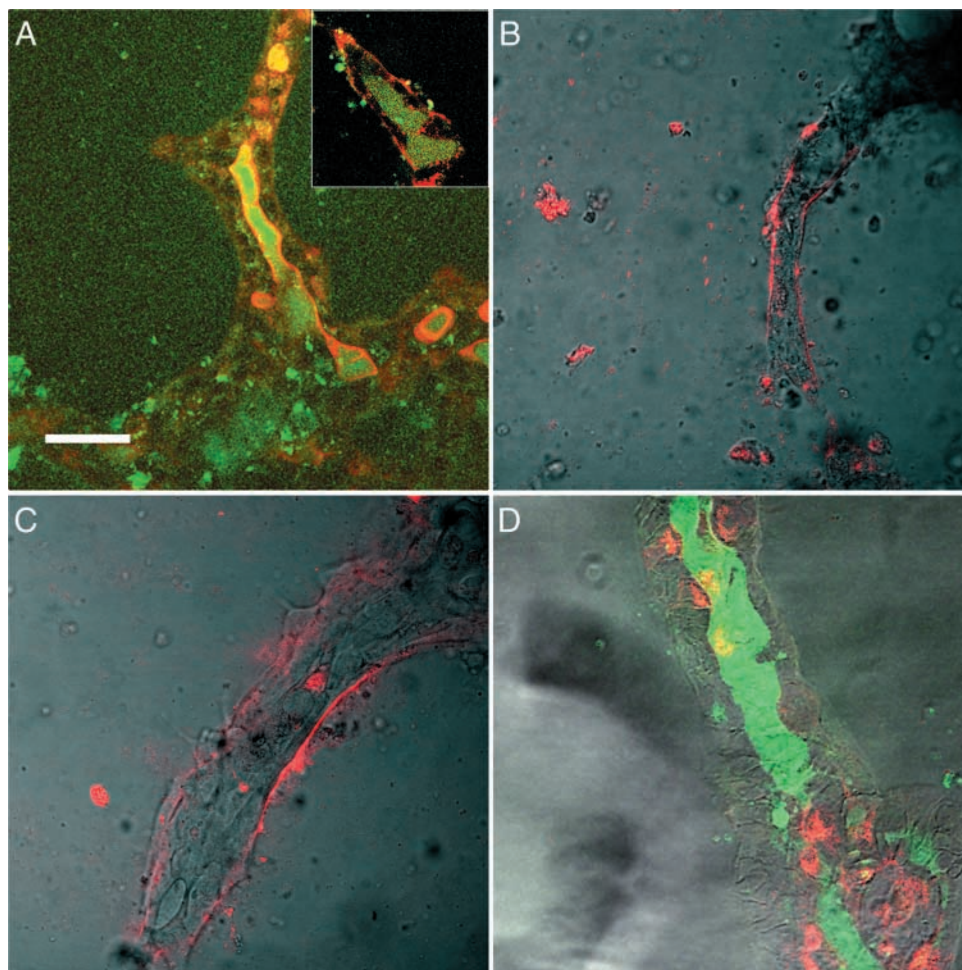

Fig. 5. Lectin binding by matrigel cultures. Matrigel cultures, initiated as described in the legend to Fig. 2 above, were incubated for 20 hours in Phenol Red-free medium supplemented with a lectin (as specified in A, B, C or D below), in addition to insulin, transferrin, hydrocortisone and Lucifer Yellow (as described in Fig. 3). Confocal images of matrigel cultures maintained 20 hours with particular lectins. (A) TRITC *T. vulgare* (30 μg/ml) (shown as red) + Lucifer Yellow (green) (insert shows a culture maintained for 20 hours as above with additional brefeldin A, 30 μg/ml), (B) FITC-Lotus *Tetragonolobus purpurea* (30 μg/ml) (green), (C) FITC-*Dolichos biflorous* (50 μg/ml) (green), and (D) TRITC-glycine max (30 μg/ml) (red) + Lucifer Yellow (green) are illustrated, as overlayed on bright-field images. The lumenal space shown in A is apparently still developing, not being contiguous, as indicated by the TRITC *T. vulgare* staining of the apical membrane, which sections off a portion of the lumen from the rest of the lumenal space. Bar in A, 50 μm (same scale for the insert in A).

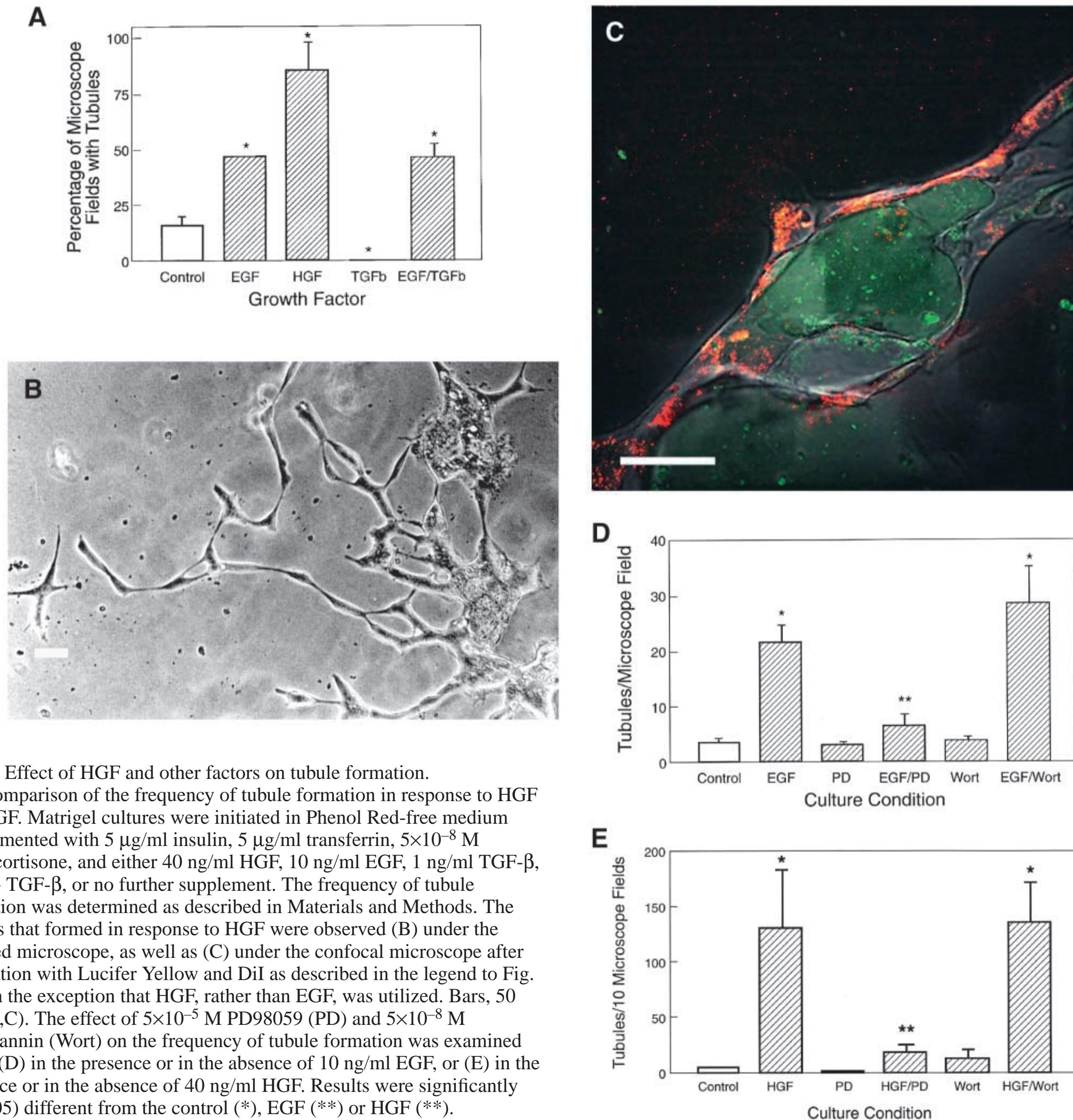

Fig. 6. Effect of HGF and other factors on tubule formation. (A) Comparison of the frequency of tubule formation in response to HGF and EGF. Matrigel cultures were initiated in Phenol Red-free medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone, and either 40 ng/ml HGF, 10 ng/ml EGF, 1 ng/ml TGF-β, EGF + TGF-β, or no further supplement. The frequency of tubule formation was determined as described in Materials and Methods. The tubules that formed in response to HGF were observed (B) under the inverted microscope, as well as (C) under the confocal microscope after incubation with Lucifer Yellow and DiI as described in the legend to Fig. 3, with the exception that HGF, rather than EGF, was utilized. Bars, 50 μm (B,C). The effect of 5×10^{-5} M PD98059 (PD) and 5×10^{-8} M wortmannin (Wort) on the frequency of tubule formation was examined either (D) in the presence or in the absence of 10 ng/ml EGF, or (E) in the presence or in the absence of 40 ng/ml HGF. Results were significantly (P<0.05) different from the control (*), EGF (**) or HGF (**).

to possessed hollow lumens (Fig. 8c). While cyst formation was stimulated in this manner (Fig. 8d), tubules were notably absent in 8-Br-cAMP-treated matrigel cultures (Fig. 8e). However, the results obtained with phorbol 12-myristate 13-acetate (TPA), an activator of protein kinase C (Fig. 8e), indicated that all agents that stimulated the process of cyst formation did not necessarily inhibit the process of tubulogenesis.

Possibly, the stimulatory effect of 8-Br-cAMP on rabbit renal cyst formation in matrigel may be due in part to a stimulatory effect of 8-Br-cAMP on growth. However, Table 1 shows that 8-Br-cAMP was not only inhibitory to the growth of monolayer cultures of primary RPT cells, but in addition, 8-Br-cAMP prevented the growth stimulatory effect of EGF.

Renal cyst formation may be the consequence of an increased rate of ion transport into the lumen of developing tubules. As the Na,K-ATPase may provide the driving force for the lumenal accumulation of such ions, the possibility that 8-Br-cAMP caused an increase in the level of expression of the Na,K-ATPase was examined by northern analysis. The results indicate that indeed, in matrigel cultures treated with 8-Br-cAMP, the levels of the mRNAs for the Na,K-ATPase α and β subunits were 2.0- and 6.1-fold higher, respectively, than in control matrigel cultures (Fig. 8f). The level of the mRNA for a marker specific for the renal proximal tubule, mitochondrial

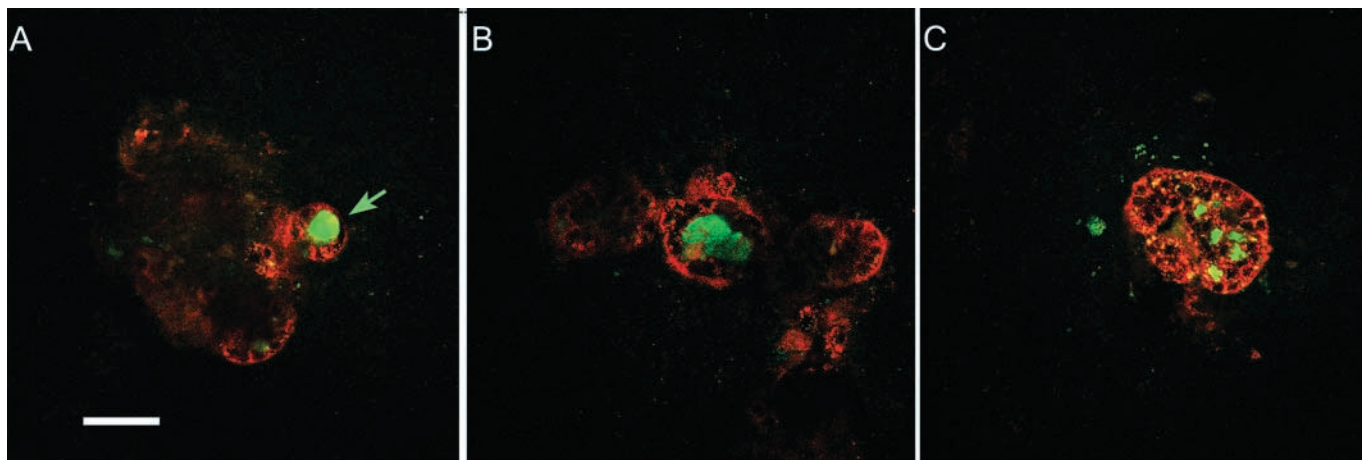

Fig. 7. Effect of PD98059, wortmannin and rapamycin on tubule formation. Matrigel cultures were maintained either with 5×10^{-5} M PD98059, 5×10^{-8} M wortmannin or 2.5×10^{-8} M rapamycin in addition to insulin, transferrin, hydrocortisone and EGF for 2 weeks. Before confocal microscopy, the cultures were maintained for 20 hours under the same conditions, with the addition of 80 μM Lucifer Yellow and 4 μg/ml Texas Red-labeled *T. vulgare*, and then examined under the confocal microscope. (A) PD98059; (B) wortmannin; (C) rapamycin. Bar in A, 50 μm (for B and C).

phosphoenolpyruvate carboxykinase (mPEPCK) (Wang and Taub, 1991), was also examined. The PEPCK mRNA level was elevated 2.3-fold in the presence of 8-Br-cAMP, unlike the case with β-actin.

It is possible that the increase in the level of expression of the Na,K-ATPase in 8-Br-cAMP-treated matrigel cultures was associated with increased organic anion transport. Indeed, the process of organic anion transport into the basolateral membrane or renal proximal tubule cells has been shown to depend on Na,K-ATPase activity (Spencer et al., 1979). Nevertheless, when examining the renal cysts which formed in 8-Br-cAMP treated matrigel cultures, Lucifer Yellow accumulation was not observed in the lumen of such cysts (Fig. 8c).

Discussion

This report presents evidence that rabbit kidney proximal tubules not only form in vitro in matrigel, but become functional. When the same cells are cultured on a polystyrene substratum, monolayers rather than tubules form (Chung et al., 1982). In vivo renal proximal tubules possess the capacity not only for reabsorption, but also for secretion of organic anions and cations. The process of organic anion secretion is initiated by the transport of organic anions present in the interstitial space into renal proximal tubule cells by means of the PAH transport system, localized on the cells' basolateral surface. Subsequently, the organic anions are extruded from the cells' apical surface into the lumen of the nephron. By means of fluorescence microscopy, we have observed that two substrates of the PAH transport system, Lucifer Yellow and 5,6-FAM, are indeed concentrated in the lumen of tubules in matrigel.

Previously, evidence has been obtained for the developmental regulation of the expression of this organic anion transport system (Pavlova et al., 2000). During murine development transcripts encoding for representative organic ion transporters (including OAT1, Roct and OAT2) appear in the kidney during midgestation, coinciding with proximal tubule differentation, and gradually increase during nephron maturation. These transporters have been proposed to play a role in the formation and maintenance of the renal proximal tubule, through the transport of organic molecules involved in morphogenesis (Pavlova et al., 2000). Growth factors probably play a role in these events. Indeed, EGF has been reported to enhance the transepithelial secretion of PAH across Opossum Kidney (OK) cells via stimulation of basolateral uptake, without affecting the apical efflux rate (Sauvant et al., 2001).

Apparently, the renal 'tubules' that form initially are cords of cells, which then form partial lumens separated from one another by cellular 'apical' membranes (as indicated by their positive staining by *T. vulgare*). Thus, although evidence has been presented using the MDCK cell line that the establishment of apicobasal polarity is a prerequisite for the formation of 'pockets of fluid-filled lumen', the formation of a mature apical membrane is not necessarily sufficient for complete lumen formation. Possibly, the 'apical' membranes surrounding the developing lumens are protrusions from the apical membrane, which are the result of chemotaxis, cytokinesis or even membrane fusion. In any case, subsequently complete lumens do form within these premature tubules. Ultimately, the majority of the tubules were observed to possess lumens and transport capacity.

In addition to our evidence for transepithelial transport via the PAH transport system, we examined the possibility that fluorescent substrates of the multidrug resistance-1 (MDR-1)-encoded pgp transport system were concentrated in the tubule lumens in matrigel. Indeed, pgp has been observed to facilitate drug secretion into the lumen of the renal proximal tubule. Although evidence for the accumulation of the MDR substrate Rhodamine 123 into the lumen of tubules in vitro, another substrate, BODIPY verapamil, only accumulated intracellularly. Presumably then, pgp-mediated active transport was occurring in the cultures, without electrodiffusive anion transport.

In addition to our confocal microscopy results with fluorescent substrates of transport systems, our results with

lectins also strongly suggest that the tubules that form in matrigel resemble renal proximal tubules. The rabbit renal tubules that formed in matrigel bind to lectins, which are known to specifically recognize rabbit renal proximal tubules, including *T. vulgare*, and Lotus *Tetragonolobus purpurea* (i.e. winged or asparagus pea lectin), while no lectin binding was obtained with Glycine max (i.e. soybean lectin). Indeed, *T. vulgare* and Lotus *Tetragonolobus purpurea* bind primarily to the apical surface of rabbit renal proximal tubule cells, whereas Glycine max stains all rabbit renal nephron segments except the proximal tubule (Le Hir and Dubach, 1982). The lectin *Dolichos biflorous*, which recognizes sugars with the specificity α-D-galNac (Holthofer et al., 1981), also bound extensively to the renal proximal tubules in matrigel. However,

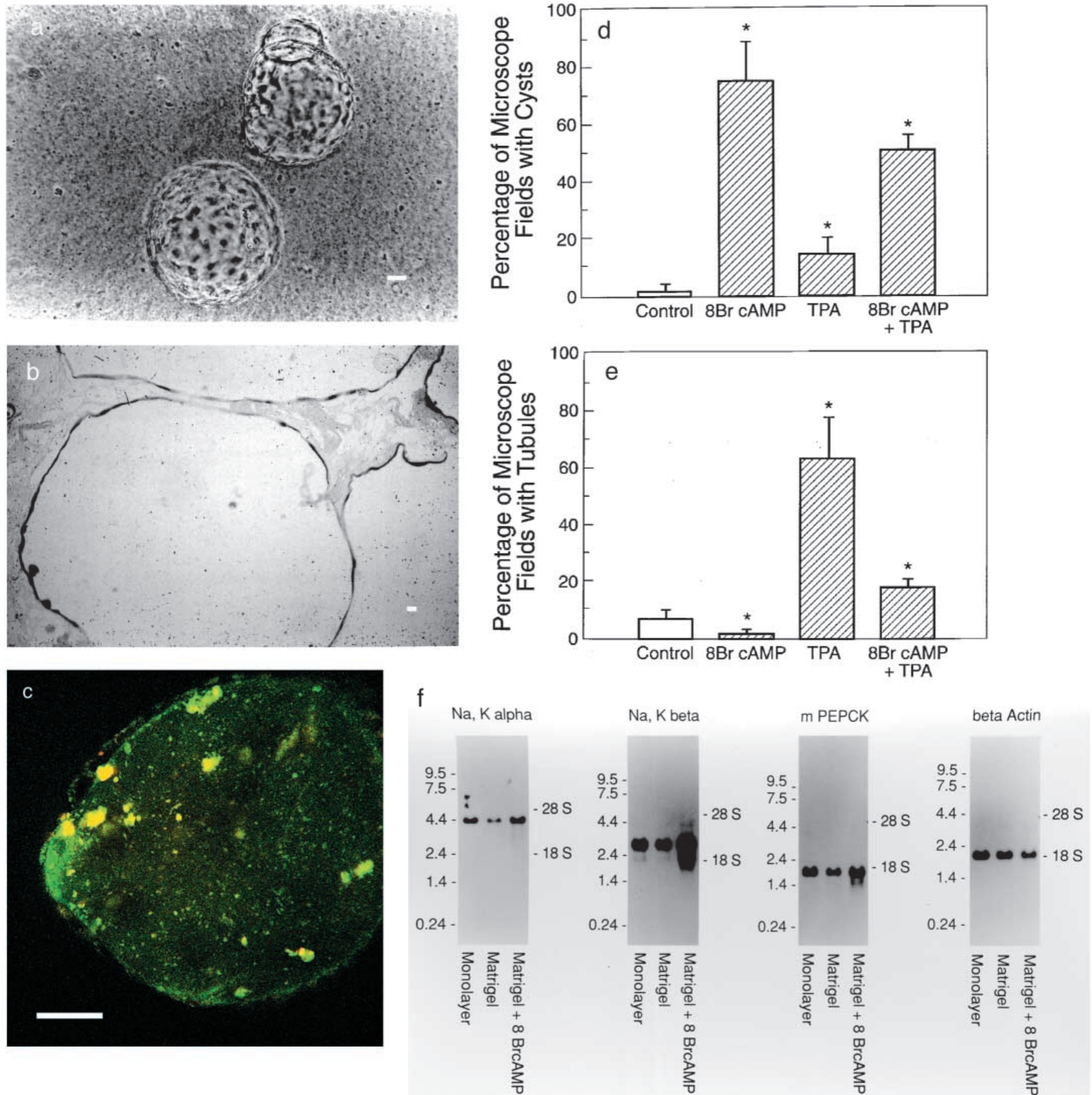

Fig. 8. Cyst formation by matrigel cultures. Matrigel cultures incubated with 8-Br-cAMP were examined (a) under the inverted microscope, (b) after the preparation of paraffin sections, as well as (c) under the confocal microscope following incubation with Lucifer Yellow. Bars, 50 μm (a-c). The effects of 8-Br-cAMP and TPA on (d) cyst formation and (e) tubule formation were quantitated as described in Materials and Methods. An effect was found to be significant (*) when $P<0.05$. (f) Effect of 8-Br-cAMP on the level of the mRNA for the Na,K-ATPase α subunit, the Na,K-ATPase β subunit, mitochondrial PEPCK, and β-actin was determined by northern analysis.

the specificity of binding of *Dolichos biflorous* for the rabbit renal proximal tubule in particular has not been defined. Other pertinent evidence that the tubules that formed in matrigel were proximal was obtained from our northern analysis, which indicated expression of mPEPCK mRNA, another specific marker of the rabbit renal proximal tubule (Vandewalle et al., 1981).

Here, we observed that the formation of rabbit renal tubules with functional lumens was a growth factor-dependent process. The emergence of functional tubules (as assessed by the accumulation of Lucifer Yellow in the tubule lumen) was observed in cultures treated with either EGF or HGF. Tubule formation did not occur in cultures treated with TGF-β alone. Previously, we similarly observed a stimulatory effect of EGF on tubule formation by primary baby mouse kidney cells in matrigel (Taub et al., 1990). Since this initial report, EGF has similarly been found to stimulate the differentiation of the whole mouse metanephroi in vitro (Perantoni et al., 1991). Branching morphogenesis from the rat ureteric bud (the collecting duct anlagen) was stimulated by EGF in the absence of the normal inducer, the metanephrogenic mesenchyme. Also consistent with the in vivo significance of these results, EGF, TGF-α and the EGF receptor have been localized both to the mesonephros and metanephros in the embryonic mouse (Bernardini et al., 1996).

EGF has been implicated as playing a major role in the morphogenesis in several other tissues. In addition to stimulating mouse mammary ductal branching morphogenesis (Coleman et al., 1988), EGF has been implicated in lung development. Indeed, neonatal mice deficient in the EGF receptor showed evidence of lung immaturity, including impaired branching, as well as deficient alveolization and septation (Kheradmand et al., 2002; Miettinen et al., 1997). The elaborate branching pattern of the *Drosophila* tracheal system has been attributed in part to activation of the EGF receptor pathway (Glazer and Shilo, 2001). Branching morphogenesis from fetal mouse submaxillary gland rudiments has been observed to be EGF responsive (Kashimata et al., 2000).

In addition to the stimulatory effects of EGF, we observed the effects of HGF on tubule formation by primary RPT cells in matrigel. Previously, tubulogenesis was observed to occur when MDCK cells were placed in a collagen gel (Montesano et al., 1991). In this study tubulogenesis by MDCK cells in a collagen gel was found to be dependent on the presence of exogenous HGF in the culture medium. Subsequently, both HGF and EGF were reported to stimulate branching morphogenesis of the mouse inner medullary collecting duct cell line mIMCD-3 in type I collagen gels (Karihaloo et al., 2001). HGF was also reported to stimulate tubulogenesis by primary rat epithelial cells grown in collagen gels (Sakurai et al., 1997). However, immortalized cells from embryonic kidneys of met –/– mice (defective in HGF signaling) were observed to retain their capacity to form tubules in vitro in response to EGF, suggesting that HGF signaling was not essential for epithelial cell development in the embryonic kidney (Sakurai et al., 1997).

Although the signaling pathways by which EGF and HGF stimulate tubulogenesis in renal cells have not yet been completely defined, our observation that PD98059 inhibits the formation of rabbit renal proximal tubules in matrigel in response to both EGF and HGF suggests the involvement of p42/44 MAP kinase. Our results suggest furthermore that the inhibitory effect of PD98059 on tubulogenesis is not simply a consequence of growth inhibition by PD98059, as PD98059 alone did not prevent the growth stimulatory effect of EGF on primary RPT cells in monolayer culture. Although PD98059 inhibited tubule formation to a large extent, our confocal microscopy studies indicated that nevertheless those tubules that formed in matrigel became functional, as luminal Lucifer Yellow accumulation was observed in tubules in PD98059-treated matrigel cultures. Similarly, in studies concerning the morphogenesis of mIMCD-3 cell cultures in response to EGF, cell process formation was not inhibited by PD98059, cell process formation being the investigators' assay of tubule development. Thus, the investigators proposed that extracellular signal-regulated kinase-5 rather than p42/p44 MAP kinase was involved in morphogenesis (Karihaloo et al., 2001).

In this report, the effects of rapamycin on rabbit kidney tubulogenesis in matrigel were also examined. Our results indicate that rapamycin treatment resulted in the formation of solid cords consisting of multilayers of cells, rather than tubules with a single lumen. Rapamycin is a microbial natural product, which on binding to the peptidyl-prolyl isomerase FKBP12 forms a complex that inhibits mTOR. mTOR is a member of the phosphoinositide 3-kinase superfamily that regulates cell growth and differentation in response to several hormones, growth factors and nutrients (Rohde et al., 2001). Phosphorylation of p70 S6 kinase by mTOR leads to S6 kinase activation, whereas phosphorylation of 4E-BP1 by mTOR causes 4E-BP1 to lose affinity for the mRNA cap-binding protein eIF4E, facilitating initiation of translation. By effecting the initiation of translation in this manner, mTOR is able to modulate the overall program of gene expression in a very precise manner. One example is the ability of mTOR to modulate the expression of different isoforms of the CCAAT/enhancer binding protein (C/EBP) α and β, which are ultimately required for the differentiation of 3T3-L1 cells into adipocytes (Calkhoven et al., 2000).

Unlike EGF and HGF, 8-Br-cAMP stimulated the formation of multicellular cysts in the primary RPT matrigel cultures. Stimulation of cyst formation by 8-Br-cAMP was associated with increased levels of the mRNAs for the α and β subunits of the Na, K-ATPase, as well as PEPCK mRNA. These observations suggest the involvement of the Na, K-ATPase in mediating the fluid secretion that occurs in these renal cysts.

Similarly, in intact renal cysts excised from the kidneys of humans with autosomal dominant polycystic kidney disease (ADPKD), the rate of fluid secretion into the cysts was observed to increase following stimulation by forskolin (an activator of adenylate cyclase) (Grantham et al., 1995). Ouabain inhibited fluid secretion. In a like manner, epithelial cell cultures derived from renal cysts, and even MDCK cells have been observed to form cysts within collagen gels in response to secretagogues which increase intracellular cyclic AMP levels. The cyclic AMP-mediated fluid secretion that occurs within such cysts has been proposed to be driven by such chloride transporters as the cystic fibrosis transmembrane conductance regulator (CFTR), as well as the Na,K, 2Cl cotransport system, in those tubular epithelial cells that possess these transport systems (Wallace et al., 1996). Our results also

indicate the involvement of the Na,K-ATPase as part of the overall phenotypic response to cyclic AMP, leading to the formation of cysts, rather than tubules. In addition, changes may occur in cell growth, the composition of the basement membrane and junctional complexes, as well as in the apical membrane itself. Indeed, the cilia protruding from the apical surface have recently been proposed to monitor the process of renal tube expansion, by changes in Ca^{2+} influx (Praetorius and Spring, 2001).

In conclusion, tubulogenesis is a complex process occurring in a variety of different types of epithelial and endothelial cells, including the kidney. Despite divergent mechanisms, common requirements for such a process appear to be initiated through the interaction of growth factors and basement membrane proteins with specific receptors localized on the cells' basolateral membrane. Needless to say, these processes are highly complex, and subject to modification by matrix metalloproteinases, which are critical to the process of branching. Presumably, cell adhesion to the basement membrane through integrins initiates signals, which in close combination with those signal generated by growth factors promote a network of processes resulting in tubule formation. Further studies will permit us understand the precise mechanisms by which growth factors and basement membrane proteins cause renal proximal tubules to acquire the capacity for transepithelial solute transport. We anticipate that the acquisition of the capacity for transepithelial transport is intimately associated with the process of the lumen formation.

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