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Expression of epidermal growth factor in the rat kidney

An immunocytochemical and in situ hybridization study

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Summary. The renal localization and the site of synthesis of epidermal growth factor (EGF) were investigated in the rat kidney by immunohistochemistry and in situ hybridization techniques. EGF was localized in the cells of the thick ascending limb of Henle (TAL) and distal convoluted tubule (DCT). At the ultrastructural level, EGF immunoreactivity was distributed on the apical membrane and trans-Golgi complex of the TAL and DCT cells. These segments of the rat nephron also hybridized to prepro-EGF cRNA probes in a specific manner, indicating that TAL and DCT are the sites of EGF synthesis in the rat kidney.

Introduction

Epidermal growth factor (EGF) is an acid stable polypeptide that is widely known to act as a mitogen as well as a differentiation factor for a wide variety of cell types (Carpenter and Cohen 1979). This factor has been identified in tissues and body fluids of several mammalian species (Hirata and Orth 1979a, b; Moore 1978; Rubin and Bradshaw 1984; Simpson et al. 1985). In all species studied, urine has been reported to contain EGF in relatively high concentrations (Fisher and Lakshmanan 1990). Considerable evidence suggests that urine-EGF originates from the kidney (Atkin et al. 1990; Fisher et al. 1989; Salido et al. 1989). Prepro-EGF mRNA and EGF-like immunoreactivity are localized to cells of the distal convoluted tubule and the thick ascending limb of Henle in the adult mouse kidney (Atkin et al. 1990; Salido et al. 1989). Our electron microscopic investigation has also revealed that most of EGF immunoreactivity is localized at the luminal membrane and apical vesicles of the tubular cells, suggesting that EGF is synthesized and stored as a transmembrane pro-EGF molecule in the kidney (Salido et al. 1986). Although the localization of EGF in the mouse kidney is well established, the information available on the expression of EGF in kidneys in other species, as demonstrated with immunocytochemical methods, do not conform to that in the mouse. In the rat, EGF was first reported to be immunolocalized to the afferent arteriole of the juxtaglomerular apparatus (Olsen et al. 1984), while in a more recent investigation, EGF localization was seen confined to the distal convoluted tubule (Poulsen et al. 1986). In man, one study localized EGF in the proximal tubule (Poulsen et al. 1986) while in another investigation immunoreactive EGF was found diffusely in the interstitium of the renal medulla (Kasselberg et al. 1985). This disagreement between the localization of EGF in the mouse kidney and in other species could be not only of possible biological importance but also of practical significance since many studies aimed to elucidate the function of EGF are carried out in experimental animals other than the mouse.

In an effort to resolve the apparent discrepancy between the localization of EGF in the mouse kidney and that seen in other species, we have investigated the immunolocalization of EGF in the rat kidney at the light and electron microscopical levels, as well as the distribution of prepro-EGF mRNA by in situ hybridization.

Material and methods

Six male adult Sprague-Dawley rats (Charles River, Wilmington, Mass., USA) were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Kidneys and submandibular glands (SMG) were removed and immersed in the same fixative for 30 min. After rinsing with PBS, the tissue for immunocytochemistry was cryoprotected with PBS containing 20% sucrose for 30 min, embedded in OCT compound (Miles, Naperville, Ill., USA), and snap-frozen in liquid N_2. Eight-micrometer-thick sections were obtained with a cryostat microtome and were mounted on glass slides coated with 50 µg/ml polylysine (ICN Immunobiologicals, Costa Mesa, Calif., USA). For in situ hybridization, fixed tissue was rinsed with PBS containing 1 m*M* aurintricarboxylic

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acid, dehydrated with ethanol and embedded in paraffin. Five-micrometer-thick sections were mounted on glass slides coated with 2% aminopropyltriethoxysilane (Sigma, St. Louis, Mo., USA).

The immunocytochemical detection of EGF was performed using a 1:2000 dilution of rabbit anti-rat EGF serum and the Avidin-Biotin Immuno-Peroxidase procedure, using biotinylated goat anti-rabbit Ig serum and avidin-biotin-peroxidase complexes (Vector Laboratories, Burlingame, Calif., USA). The antiserum was generated against purified EGF isolated from rat submaxillary glands (Schaudies and Savage 1986) and its specificity of the anti-rat EGF serum was determined by radioimmunoassay and by immunoblotting (Lakshmanan et al., unpublished). The sections were incubated with the anti-rat EGF serum overnight, washed with PBS for 30 min, and incubated with 5 µl/ml of biotinylated goat anti-rabbit Ig serum for 1 h. After a 15-min wash with PBS, the slides were immersed in methanol containing 0.1% hydrogen peroxide, to inhibit the endogenous peroxidase, for 5 min. After another 15-min wash with PBS, tissue sections were incubated with avidin-biotin-peroxidase complexes for 1 h, washed for 30 min with PBS, and incubated with the substrate solution (0.5 mg/m diaminobenzidine (DAB) and 0.03% hydrogen peroxide in 50 m*M* *Tris*-HCl buffer, pH 7.6). Sections were then rinsed for 15 min in PBS, dehydrated in a graded series of alcohol solutions, and mounted. Control sections were incubated with 1:1000 dilution of non-immune rabbit serum and processed in a similar manner.

In situ hybridization analysis of pro-EGF mRNA was carried out by using antisense RNA probes generated by in vitro transcription. A 1.7 Kbp BamH1-Pst1 fragment (from base pair 1304 to 3062) of the rat pro-EGF cDNA (Dorow and Simpson 1988) was subcloned into the BamH1-Pst1 site of the RNA transcription vector pGEM-4 (Promega, Madison, Wisc., USA) using T4 DNA ligase and standard cloning procedures (Maniatis et al. 1982). The plasmid was linearized by using either EcoR1 or Hind3 to generate appropriate templates for in vitro transcription of pro-EGF antisense RNA (cRNA) and pro-EGF sense RNA, respectively. The in vitro transcription was carried out using either T7 or SP6 RNA polymerase (Promega) to generate cRNA or sense RNA, respectively, according to previously published methods (Melton et al. 1984). ^{35}S-labeled CTP (100 pmol sp act 1400 Ci/mmol, Amersham Corp., Arlington Heights, Ill., USA) was included in the nucleotide mixture. The specificity of the reaction was determined by autoradiography of 1 µl of the transcription product separated in formaldehyde-agarose gel (Maniatis et al. 1982).

Tissue sections were dewaxed, hydrated, and treated with 1 µg/ml proteinase K (Sigma) for 7 min at 37° C. After rinsing with PBS containing 2 µg/ml of glycine (Sigma) sections were post-fixed with 4% paraformaldehyde for 10 min, and sequentially rinsed with PBS containing 2 µg/ml of glycine, and 0.2 × saline sodium citrate (SSC). The slides were drained and incubated at 37° C for 2 h in a prehybridization mixture containing 50% formamide, 0.75 *M* NaCl, 25 m*M* piperazine-*N*, *N'-bis*-2ethanesulfonic acid (pH 6.8), 25 m*M* EDTA, 1 × Denhardt's, 100 m*M* dithiothreitol (DTT), 0.2% sodium dodecyl sulfate (SDS), and 300 µg/ml salmon sperm DNA. Next, the hybridization mixture (containing 3 × 10 cpm/µl of ^{35}S-labeled pro-EGF antisense-RNA in a solution identical to the prehybridization mixture with the addition of 5% dextran sulfate) was applied. In control sections ^{35}S-labeled pro-EGF sense-RNA of similar specific activity was used. Siliconized cover slips were placed on the sections with the hybridization mixture, and the hybridization reaction was carried out at 45° C for

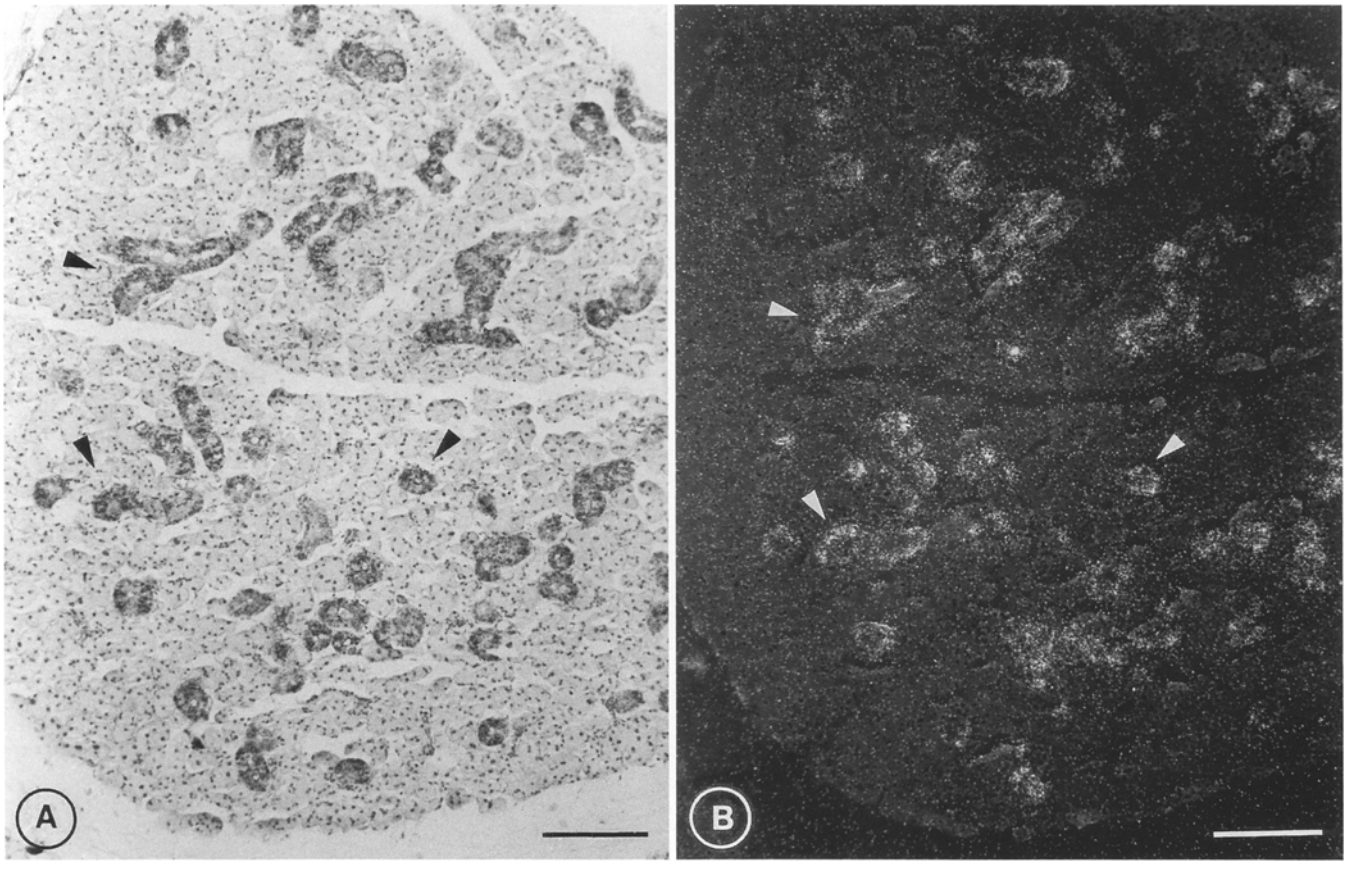

Fig. 1A, B. Expression of EGF in the rat submandibular gland. Granular convoluted tubules (*arrowheads*) show intense EGF immunoreactivity (**A**) and prepro-EGF mRNA hybridization (**B**) in adjacent serial sections of paraffin embedded material. No signals are evident in the acini of the gland. *Bar* = 150 µm

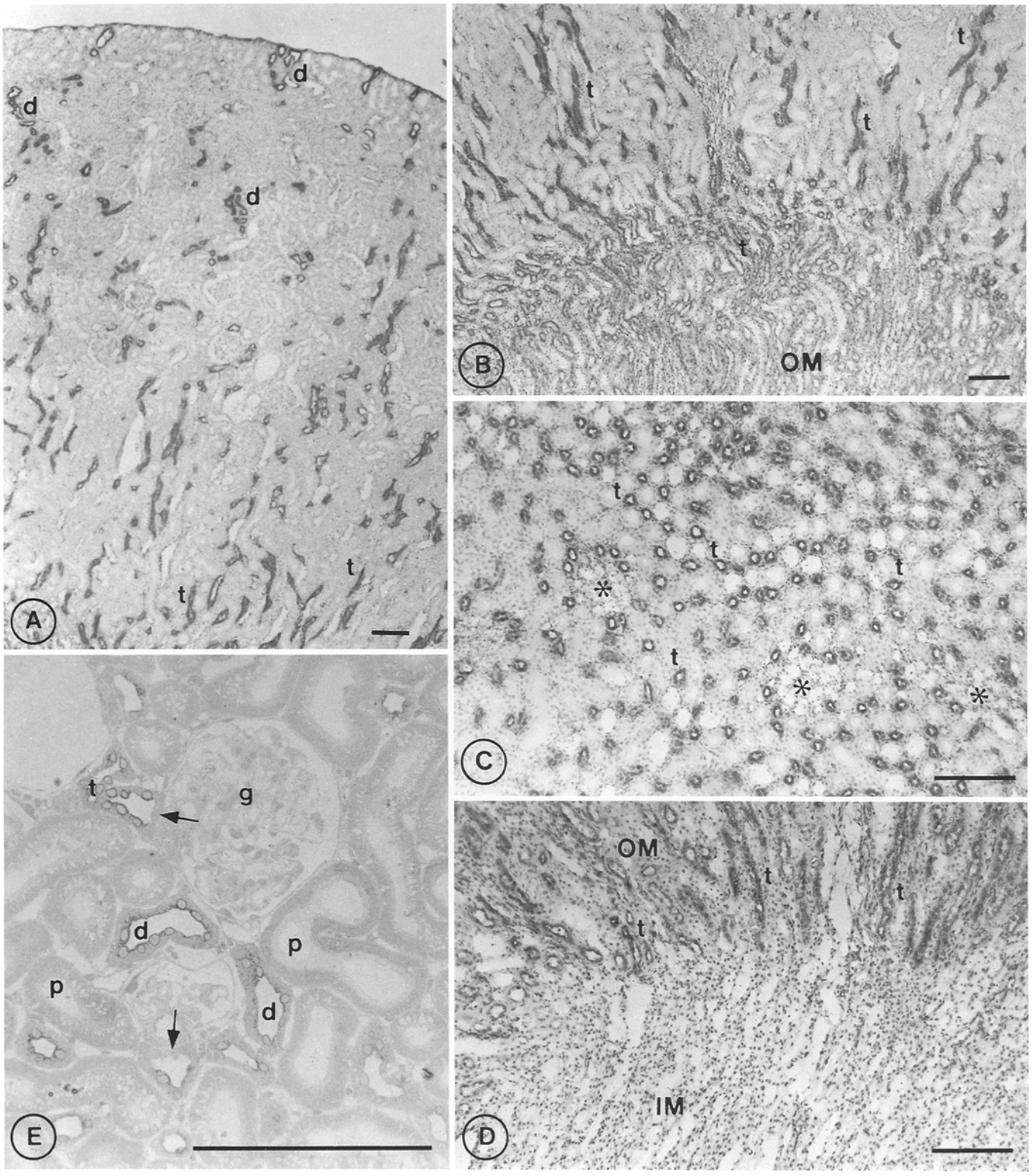

Fig. 2A–D. EGF immunoreactivity in the rat kidney. **A** Renal cortex: dense immunostaining is observed in the distal convoluted tubules (*d*) and thick ascending limb of Henle (*t*). **B** Juxtamedullary zone: EGF immunoreactivity is shown in the thick ascending limb of Henle (*t*) at the outer medulla (*OM*). **C** Crosssection at the outer medulla: thick ascending limbs of Henle (*t*) show abundant EGF immunoreactivity and a characteristic distribution around vascular bundles (*asterisk*). **D** Renal medulla: EGF-containing thick ascending limbs of Henle (*t*) are observed in the outer medulla (*OM*), while the inner medulla (*IM*) lacks EGF immunoreactivity. **E** High resolution immunocytochemistry: apical membrane distribution of EGF immunoreactivity is present in the distal convoluted tubules (*d*) and thick ascending limbs of Henle (*t*). No EGF is detected in proximal tubules (*p*), glomeruli (*g*), or juxtaglomerular apparatus. The macula densa (*arrows*) lacks EGF immunoreactivity in clear contrast with the adjacent cells of the TAL, which are notably positive. *Bars* = 150 μm

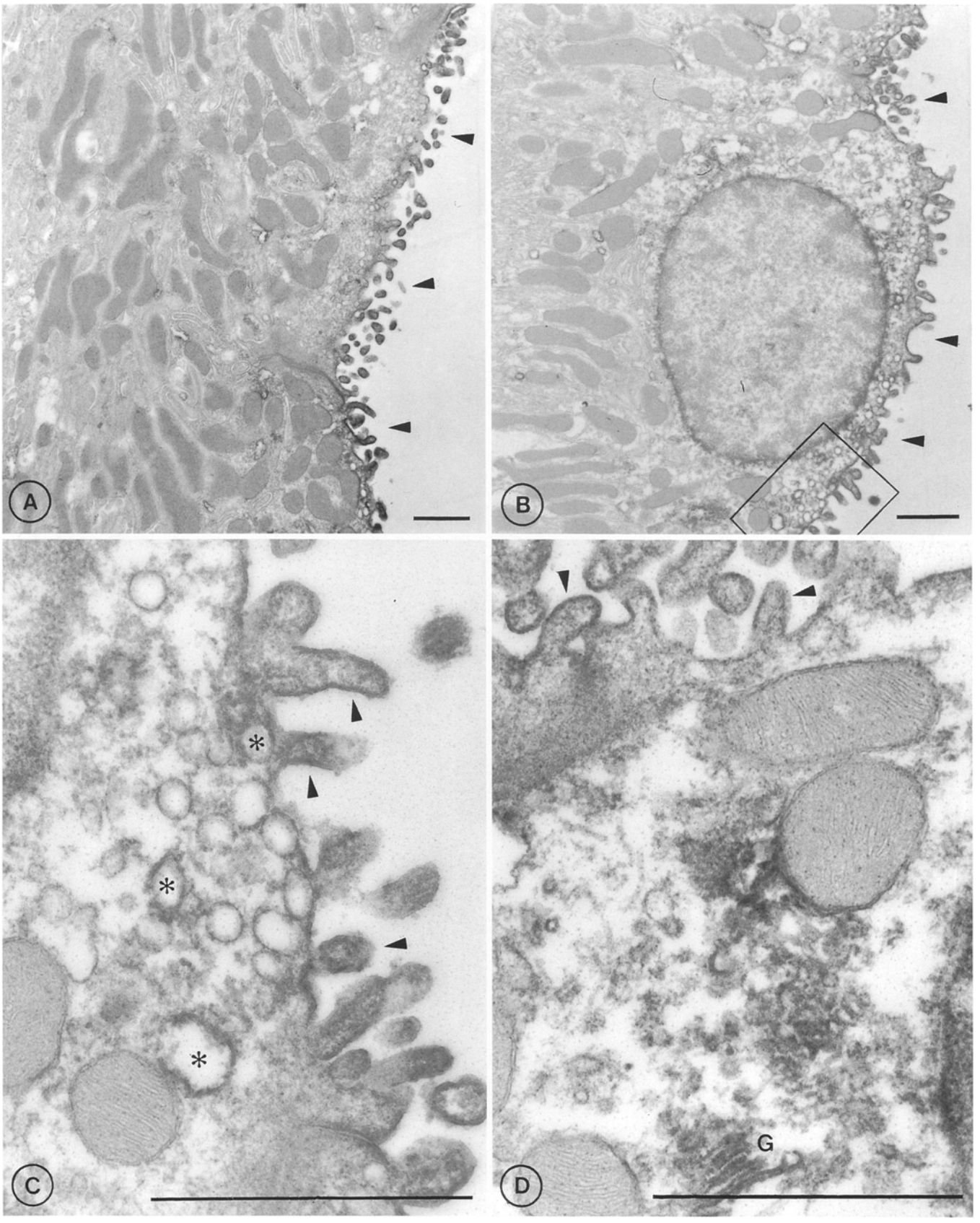

Fig. 3A–D. Immuno electron microscopy of EGF in the rat kidney. **A** Thick ascending limb of Henle: EGF immunoreactivity (*arrowheads*) is present along the apical membrane. **B** Distal convoluted tubule: EGF immunoreactivity is shown on the apical membrane (*arrowheads*). **C** Higher magnification of the distal convoluted tubule cells shown in **B**: numerous EGF-positive vesicles (*asterisks*) are distributed in the apical portion of the cytoplasm. **D** Higher magnification of the distal convoluted tubule cell: vesicles associated to the trans-Golgi complex (*G*) show EGF immunoreactivity along with the apical membrane (*arrowheads*). *Bar* = 1 μm

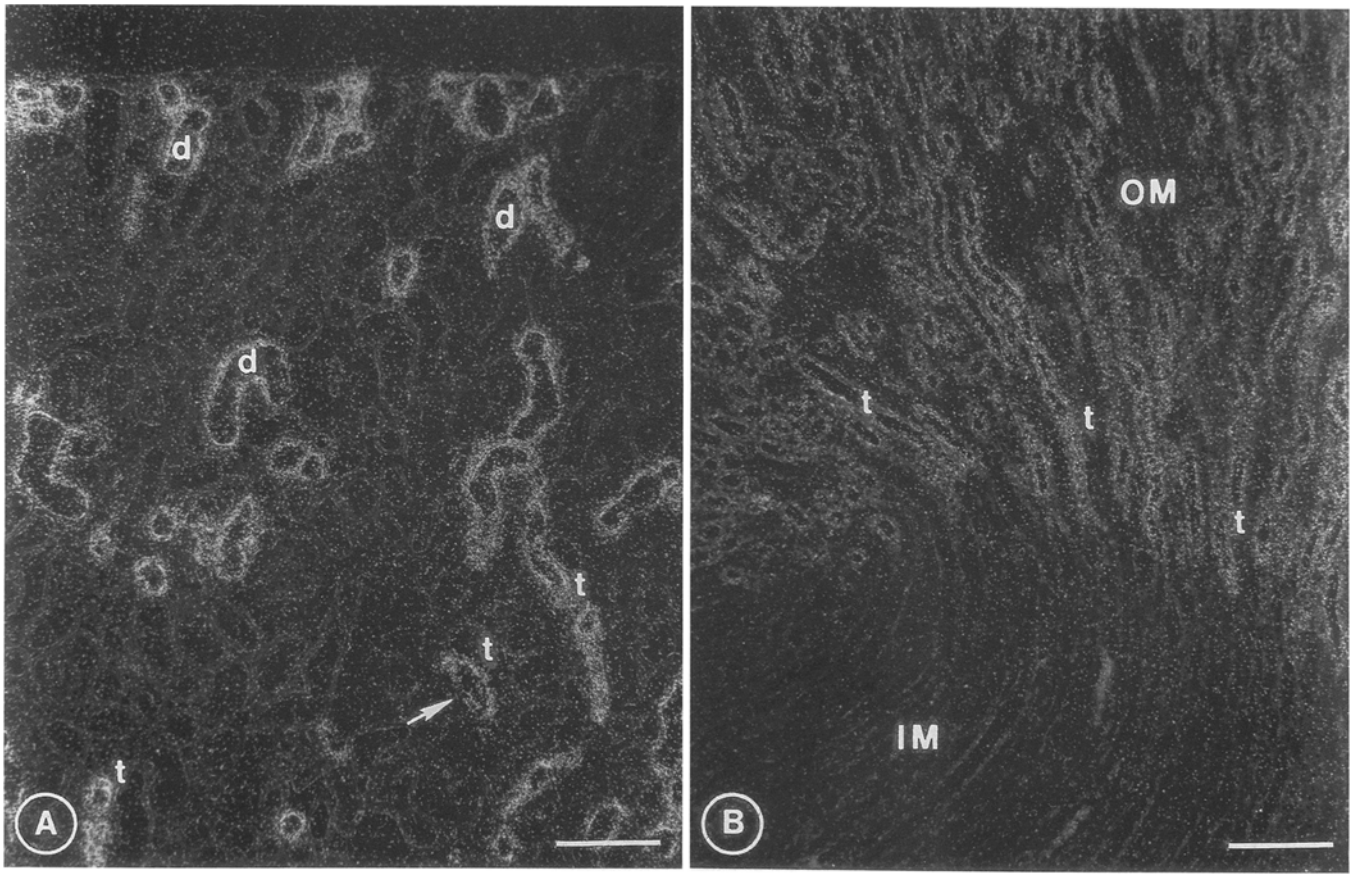

Fig. 4A, B. In situ hybridization of prepro-EGF mRNA in the rat kidney. ^{35}S-labelled pro-EGF cRNA hybridizes to numerous tubular profiles in both cortical – **panel A** – and outer medullary (*OM*) regions – **panel B** – corresponding to the distal convoluted tubules (*d*) and thick ascending limbs of Henle (*t*). The inner medullary region (*IM*) is negative. The macula densa (*arrow*) lacks pro-EGF cRNA hybridization, while the rest of the TAL at this level is positive. *Bar* = 150 μm

12 h. After hybridization, cover slips were removed and slides were washed with 4 × SSC solution prior to ribonuclease A digestion. This was performed by incubating the slides for 30 min at 37° C with 25 μg/ml ribonuclease A (Sigma) in 0.5 *M* NaCl, 10 m*M* *Tris*-HCl (pH 8.0), 1 m*M* EDTA, 10 m*M* sodium thiosulfate. Next, the slides were washed with the same buffer without the ribonuclease A for 30 min at room temperature and then for 30 min with 2 × SSC and 10 m*M* sodium thiosulfate at room temperature. A final high-stringency wash was carried out with 0.1 × SSC and 5 m*M* sodium thiosulfate at 45° C for 60 min. The sections were dehydrated in a graded series of alcohol solutions and vacuum dried. The sections were dipped in a 50% aqueous solution of photographic emulsion NTB-2 (Eastman Kodak, Rochester, NY) at 45° C, exposed for 7–10 days at 4° C in bakelite boxes containing desiccant, developed with D19 developer (Eastman Kodak) and fixed with Rapid Fix (Eastman Kodak). The sections were counterstained with hematoxylin, dehydrated, and mounted.

Sections were examined and photographed with a Zeiss (Oberkochen, FRG) Axiophot photomicroscope using bright field and dark field condensers.

For immuno-electron microscopy, the samples were fixed with 4% paraformaldehyde containing 0.01% glutaraldehyde in PBS. Seventy-five-micrometer-thick vibratome sections were incubated with a 1:1000 dilution of the anti-rat EGF serum overnight and the avidin-biotin-immunoperoxidase procedure was performed as above, but a 1% glutaraldehyde post-fixation step was introduced after the avidin-biotin-peroxidase incubation. The slides were dehydrated and embedded in Epon resin. The blocks were sectioned for high resolution light microscopy and electron microscopy. Thin sections were observed and photographed in a Siemens electron microscope.

Results

The anti-rat EGF antisera reacted specifically with the granular convoluted tubules of the SMG, as determined by avidin-biotin immunoperoxidase (Fig. 1 A). The same cells also hybridized with ^{35}S-labeled pro-EGF cRNA, as shown by in situ hybridization of adjacent serial sections (Fig. 1 B). SMG acini and ducts lacked both EGF immunoreactivity and pro-EGF mRNA (Fig. 1). Control sections treated with either non-immune rabbit serum or sense probe showed no immunostaining or hybridization, respectively (data not shown).

In the kidney, both EGF immunoreactivity and prepro-EGF mRNA were localized in tubular profiles of the cortex and outer zone of the medulla. Distal convoluted tubules (DCT) in the cortex of the rat kidney showed intense EGF immunoreactivity (Figs. 2 and 3) as well as abundant hybridization with pro-EGF cRNA probe (Fig. 4). In addition, the cortical thick ascending limb of Henle (cTAL) showed both EGF immunoreactivity and pro-EGF mRNA (Figs. 2 and 4). However, the macula densa cells did not show EGF or EGF

mRNA, whereas the rest of the cells of the TAL at this level were positive (Figs. 2A and 4A). The outer zone of the medulla was rich in TAL (mTAL) that reacted specifically with anti-EGF serum (Fig. 2B–D) and pro-EGF cRNA (Fig. 4B). EGF-expressing mTALs were observed in a characteristic peri-vascular pattern in cross-sections at the transition between the outer and inner stripes of the outer zone of the medulla (Fig. 2C). No EGF immunoreactivity or pro-EGF mRNA was detected in the inner zone of the medulla (Figs. 2D and 4B). Neither the anti-EGF serum nor the pro-EGF probe labeled other portions of the nephron, vasculature or interstitium.

At the ultrastructural level, EGF immunoreactivity was localized on the apical membrane of the DCT and TAL cells, as well as the vesicles of the trans-Golgi complex (Fig. 3). Most of the EGF-positive vesicles were observed in the supra-nuclear area of the cytoplasm, close to the apical membrane of the cells.

Discussion

The EGF antiserum used in the present study was generated against an electrophoretically pure EGF preparation isolated from adult male rat SMG. The antiserum, at 1:1000 dilution, showed a single immunostainable peptide in the 6 kDa region when reacted with 20 μg of purified EGF on Western blot analysis, indicating that the preparation used for immunization was free of significant contaminants (Lakshmanan J., et al., unpublished). In rat SMG, the antiserum produced strong immunostaining of the granular convoluted cells, which also showed intense hybridization signals for pro-EGF mRNA. The parallel distribution of pro-EGF mRNA signals and EGF immunostaining in the granular tubule cells is consistent with the view that these cells are the site of EGF synthesis and storage in the SMG. Similar findings have been reported regarding EGF expression in mouse SMG (Gresik et al. 1985).

In the kidney, EGF immunoreactivity was observed in medullary and cortical regions, corresponding to the thick ascending limb of Henle (TAL) and distal convoluted tubule (DCT). The macula densa was consistently negative for EGF immunostaining. No EGF-positive immunostaining was observed in other regions of the nephron, vasculature or interstitium. In particular, both afferent and efferent arterioles showed no EGF immunoreactivity. This finding is in contrast with a previously reported study of EGF in the rat kidney, where immunostaining was localized in the renin-containing cells of the juxtaglomerular apparatus (Olsen et al. 1984). In another immunohistochemical study, EGF immunoreactivity was reported to be present in the distal convoluted tubule (Poulsen et al. 1986). The reason for the discrepancy between these two reports is not clear. In the present study, intense hybridization signals were observed in the cells of the TAL and DCT, the same portions of the nephron that showed EGF immunoreactivity. The absence of EGF expression in the macula densa cells, even when the rest of the cells of the TAL at this level are strongly positive, is a consistent finding of unknown significance. A similar situation has been reported previously in the mouse kidney (Atkin et al. 1990; Salido et al. 1986). The present results, together with similar observations in mouse kidney, convincingly demonstrate that TAL and DCT are the sites of EGF expression in both rat and mouse.

At the electron microscopic level, the EGF immunoreactivity was localized to the apical membrane and vesicles of the trans-Golgi complex from both TAL and DCT cells. Vesicles in the supranuclear area, some in close association with the apical membrane, were rich in EGF immunoreactivity. EGF-immunoreactive vesicles were found only occasionally in basal cytoplasmic zones. The observed intracellular distribution of EGF immunoreactivity is very similar to our previous findings in mouse kidney (Salido et al. 1986), suggesting that renal tubular pro-EGF is processed similarly in the mouse and rat. The similarity in the domain distribution of mouse and rat prepro-EGF cDNA (Dorow and Simpson 1988; Rall et al. 1985; Scott et al. 1983), also supports this concept.

The cloned prepro-EGF cDNAs for mouse (Scott et al. 1983), rat (Dorow and Simpson 1988), and human (Bell et al. 1986) predict the EGF precursor in all three species to be twenty times larger than mature EGF. The predicted precursors contain a 20 amino-acid transmembrane domain and several EGF-like repeats. There are several proteolytic cleavage sites and *N*-linked glycosylation sites. If the precursors undergo proteolytic cleavage and glycosylation at these predicted sites, there is the potential to generate multiple biologically active peptides in addition to mature EGF. The ultrastructural localization of EGF immunoreactivity to the apical membrane of the TAL and DCT cells suggests that urinary EGF might be generated by cleavage of membrane-bound EGF precursor by proteases present in the tubular fluid. At the present time, there is little information regarding the intracellular and membranous processing of the EGF-precursor in mouse, rat, or man. Based on the metabolic studies with mouse SMG and kidney tissue slices, Rall et al. (1985) suggested that the EGF precursor is processed to yield the 6 kDa mature EGF only in the salivary gland and was present as a 130 kDa species in kidney. Recently, Breyer and Cohen (1990) demonstrated the presence of a 140–150 kDa glycosylated membrane-associated EGF-precursor in mouse kidney. The antiserum used in our present investigation reacted strongly with both SMG-EGF and kidney-EGF, suggesting that it recognizes mature EGF as well as the EGF-precursor protein.

Membrane-bound human pro-EGF expressed in vitro is capable of binding to and activating EGF receptors (Mroczkowski et al. 1989). High affinity EGF receptors have been described in cells of the mesangium, proximal tubule and inner medullary collecting duct (Breyer et al. 1988; Gusterson et al. 1984; Harris 1989; Harris et al. 1988; Margolis et al. 1988; Norman et al. 1987; Real et al. 1986; Stanton and Seifter 1988). In these cells, EGF induces a variety of responses, including phospholipase A_2 activation (Bonventre et al. 1990; Margolis et al. 1988) and contraction (Harris et al. 1988)

of mesangial cells, inhibition of gluconeogenesis (Harris and Daniel 1989), increase of intracellular pH and enhancement of hexose monophosphate shunt activity (Stanton and Seifter 1988) in proximal tubule cells, and modulation of sodium absorption (Vehaskari et al. 1989), arachidonate metabolism (Harris 1989) and hydraulic conductivity (Breyer et al. 1988) in collecting duct cells. In addition, EGF stimulates cell proliferation and maturation in a variety of tissues and cultured cells, including renal cells (Adler and Eng 1990; Goodyer et al. 1988; Normen et al. 1987). Thus, it can be speculated that EGF could act in a paracrine fashion in the kidney.

There is evidence to suggest that EGF may play an important role in the regenerative response after renal injury (Humes et al. 1989; Norman et al. 1987; Safirstein et al. 1989). However, EGF receptors have been found in the basolateral membrane of proximal tubules and collecting ducts, and it is not obvious how EGF from the luminal membrane of the distal nephron might interact with the basolateral membrane receptors of these segments of the nephron. In situ studies on the distribution of EGF receptors in the urinary system should help clarify the conflict between the location of EGF receptors and the site of EGF synthesis in the kidney.

The rat has been widely used as an experimental animal in the investigation of the function of renal EGF (Bonventre et al. 1990; Harris 1989; Harris and Daniel 1989; Harris et al. 1988; Humes et al. 1989; Safirstein et al. 1989). We have shown that the TAL and DCT cells are the site of EGF expression in the rat kidney.

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