EGF precursor mRNA and membrane-associated EGF precursor protein in rat exorbital lacrimal gland

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Maréchal, Hervé, Hélène Jammes, Bernard Rossignol, and Philippe Mauduit. EGF precursor mRNA and membrane-associated EGF precursor protein in rat exorbital lacrimal gland. Am. J. Physiol. 276 (Cell Physiol. 45): C734–C746, 1999.—This study was designed to demonstrate the presence of epidermal growth factor (EGF) in the rat exorbital lacrimal gland. EGF precursor gene transcription was demonstrated first by RT-PCR analysis of lacrimal gland RNA using a set of specific primers and second by Northern blot analysis of rat lacrimal gland mRNA. A rabbit polyclonal antibody (rEGF2) directed against rat submaxillary gland EGF precursor protein biochemistry that growth factors of the EGF pathway where they mature through proteolytic cleavage before being released outside the cell. It is now well established from both cDNA sequence analysis and protein biochemistry that growth factors of the EGF family are also synthesized in the form of precursor molecules. However, they constitute an exception to the general model, since they are thought to be synthesized as transmembrane glycoproteins. For example, cDNA analysis of the EGF precursor predicted proteins of 1,207, 1,217, and 1,133 amino acids, respectively, for human (1), mouse (33, 40), and rat (34), resulting in glycoproteins of molecular mass between 140 and 170 kDa. This molecule is made up of a large extracellular region (~1,000 amino acids) with the EGF sequence (48–53 amino acids) located near the plasma membrane, a transmembrane domain, and an intracellular domain of variable length (19). In tissues such as kidney (2, 33, 37) and mammary gland (3), EGF is present as part of the extracellular portion of the transmembrane precursor. However, in the submaxillary gland from rodents, the EGF precursor molecule is fully and intracellularly processed into EGF (33) and stored in the secretory granules of the granular convoluted tubules (31, 39); thus the ways by which EGF is secreted in the extracellular medium must be completely different. In the first case, the precursor needs to be cleaved extracellularly by an unidentified ectoprotease (12, 13), whereas, in the second case, secretion involves the well-known regulated exocrine secretion through stored secretory granules (6).

Recently, it was shown that human (27, 49), mouse (45), and rat (50) tears contained EGF or an EGF-like immunoreactivity and that the total production of EGF in human tears increased during reflex tearing (46). The EGF precursor mRNA has been detected by Northern blot in mouse (14) and by RT-PCR in human (52) lacrimal glands. Moreover, EGF-like immunoreactivities have been detected in human (26), mouse (14), and rat (48, 50) lacrimal tissues.

The lacrimal gland produces the complex aqueous portion of tears, which contains many components, including electrolytes and proteins. The pH, electrolyte concentration, and protein composition of lacrimal fluids are crucial in maintaining the health of the ocular surface. The proteins synthesized and secreted by the lacrimal glands are very specific and are thought to be mainly involved in the bacteriostatic action of tears. Until now, only a few of them have been identified, but their secretion involves both the constitutive (42) and regulated pathways (8). From the work of Savage and Cohen (35), showing the stimulating effect of EGF on corneal epithelial cell proliferation, the role of EGF in corneal wound healing has been extensively studied. The results indicated that EGF and other growth
factors were involved in the stimulation of reepithelialization processes as well as keratinocyte and corneal endothelium proliferation (5, 51). Thus the conserved presence of EGF and/or EGF-related molecules in tears and lacrimal glands of both humans and rodents further suggested that it may serve an important function in the pericocular environment. In light of these results, it was suggested that the lacrimal gland could be important in the process of corneal wounding by producing growth factors secreted in tears (51). In the human lacrimal gland, EGF secretion could involve a muscarinically regulated pathway (53). However, until now nothing was known about the storage form of EGF in this tissue and consequently about the way by which EGF could be secreted into the tear fluid.

In view of the above hypothesis, and because the lacrimal gland tissue from rat is very easy to obtain compared with its human counterpart, we decided to look for the presence of EGF and to identify the molecular form present in the rat exorbital lacrimal gland. We first examined EGF gene expression by both RT-PCR and Northern blot analysis. Second, EGF-containing proteins were identified and localized using anti-rat EGF and anti-rat EGF precursor antibodies. Results obtained were compared with reference tissues such as rat submaxillary gland and kidney. Our results demonstrate for the first time both EGF precursor gene transcription and EGF precursor protein expression in a lacrimal tissue, i.e., the rat exorbital lacrimal gland. Contrary to previous observations made in both mouse (14) and rat (50) lacrimal gland, we were unable to detect the 6-kDa soluble form of mature EGF in the rat lacrimal gland. Our results demonstrate that EGF is stored only as its full-length membrane-associated precursor and may provide key information to study the regulation of its secretory process.

MATERIALS AND METHODS

Animals. Adult male albino Sprague-Dawley rats were obtained from IFFA CEDO and used throughout this study.

Chemicals. Peroxidase-conjugated and alkaline phosphatase-conjugated goat anti-rabbit IgG, mouse EGF (mEGF), synthetic rat TGF-α, trypsin (10,000 N-s-benzoyl-L-argnine ethyl ester units/mg), soybean trypsin inhibitor (1 mg inhibits 10,000 units trypsin), polyethylene glycol 6000, pepstatin A, leupeptin, chymostatin, antipain, p-nitrophenyl phosphate, p-nitrophenol, and protein molecular mass markers were obtained from Sigma Chemical (St. Louis, MO). Enhanced chemiluminescence (ECL) developer and Hyperfilm were obtained from Amersham France (Les Ulis, France). Bicinchoninic acid (BCA) protein assay kit and Immunopure Ag/Ab immobilization trial kit (Sigma Link coupling gel) were purchased from Pierce (Rockford, IL). Recombinant human EGF (hEGF) was from Preprotech (Washington, MA). Triton X-100 was obtained from Merck (Darmstadt, Germany). Bio-Gel P-10 was from Bio-Rad Laboratories (Ivry/seine, France). Prepack HIC Prep 16/60 Sephacryl S-200 HR, Mono Q HR 5/5, PD-10 desalting columns, deoxyxynucleotide triphosphates, protein A-Sepharose CL-4B, agarose, and DNA size markers were obtained from Pharmacia Biotech (Orsay, France). Superscript RNase H⁻ RT, random primers, and restriction enzymes Pst I, Hae III, and Sst I were from GibCO BRL (Ergany, France). The random primer DNA labeling kit was obtained from Boehringer Mannheim (Meylan, France). Upstream and downstream oligonucleotide primers and synthetic peptide p437 were synthesized by Eurogentec (Seraing, Belgium). Taq DNA polymerase was from Appligene (Illkirch, France). [125I]NaI (100 mCi/ml, 3.7 MBq/ml), [α-32P]dCTP (3,000 Ci/mmoll, 111 TBq/mmol), and 51-t-labeled mEGF (150–200 μCi/µg, 5.6–7.4 MBq/µg) were purchased from New England Nuclear (Les Ulis, France).

Rat submaxillary gland EGF purification. Rat EGF was isolated from submaxillary glands of adult male Sprague-Dawley rats (at least 12 wk old) using rapid HPLC techniques according to Simpson et al. (41). This purification involved the homogenization of the frozen tissue in drastic acidic conditions followed by centrifugation of the homogenate. The EGF contained in the soluble material was purified by sequential chromatography through a preparative reverse-phase C18 column and an analytical C18 HPLC column and anion exchange on a mono-Q column. During the course of this purification, rEGF was followed by radioreceptor assay (RRA) as described below but using 125I-labeled mEGF as radioligand. From 23 g of tissue (wet mass), we obtained ~600 µg of purified rEGF. The purity of the final product was checked by comparison with commercial mEGF and hEGF by SDS-PAGE, automated amino acid sequence analysis, and ability to stimulate HC 11 cell growth (data not shown).

rEGF radiolabeling. Native rEGF was radiolabeled with [125I]I by the chloramine T method. Briefly, 1 µg rEGF dissolved in 20 µl of 0.25 M sodium phosphate buffer (pH 7.4) was incubated for 45 s in the presence of 0.5 mCi (5 µl) carrier-free [125I]NaI and 10 µl chloramine T (2 mg/ml) at room temperature. The reaction was terminated by the addition of 20 µl sodium metabisulfite (2 mg/ml) and 40 µl NaI (2.5 mg/ml) and the mixture was diluted to 500 µl with 50 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 1% BSA. [125I]-rEGF was separated from nonincorporated [125I]I by chromatography on a PD-10 (Sephadex G-25) desalting column. In these conditions, the peptide incorporated 40–60% of the radioactivity, and the eluted iodinated peptide is 95–97% precipitable by 10% TCA.

Antibody production. Purified rEGF was used to generate polyclonal rabbit antibodies. Production was performed according to the Eurogentec custom protocol of immunization. Two rabbits were immunized with 50 µg of rEGF and further boosted twice at 15-day intervals and then once more 1 mo later. The presence of specific anti-rEGF antibodies in sera was tested by their ability to immunoprecipitate 125I-rEGF. Both rabbits produced antibodies, but only one (rEGF 2) was retained because of its higher serum titer. The IgG fraction of this antiserum was prepared by chromatography on protein A-Sepharose.

A polyclonal rabbit prepro-EGF antibody was raised against a synthetic peptide (p437; CESSKKPSEESSSN). This peptide corresponds to amino acids 1,068–1,081 of the sequence of the rat prepro-EGF and is located in the predicted intracellular region of the precursor molecule. The NH₂-terminal cysteine was added to the EGF precursor sequence to conveniently conjugate the peptide to a carrier protein, keyhole limpet hemocyanin (KLH), to improve its immunogenicity. The conjugated peptide was then used to immunize two rabbits according to Eurogentec specifications (see above). The presence of antibodies in sera was tested by ELISA using p437-coated plates. The presence of absorbed antibodies was detected with anti-rabbit IgG alkaline phosphatase-conjugated goat antibody through the hydrolysis of p-nitrophenyl phosphate as substrate. Specific antipeptide antibodies (pEPGF1) were purified by affinity chromatography from the serum containing the highest titer to eliminate all the
contaminating anti-KLH antibodies. This was achieved by chromatography through p437-conjugated agarose gel (SulfoLink coupling gel from Pierce) according to the manufacturer's recommendations.

Preparation and fractionation of the rat tissues. Rats were killed by carbon dioxide inhalation. The exorbital lacrimal, parotid, and submaxillary glands, heart, brain, kidney, and liver were rapidly removed. Glandular tissues were trimmed of their fatty and connective tissues, and hearts and livers were extensively washed to eliminate blood contamination as much as possible. All tissues were further fragmented into small pieces and either used for the preparation of lacrimal acini as described previously (20) or frozen in liquid nitrogen and stored at −20°C until processing for RNA (18) or subcellular fraction preparation (see below).

To quantify EGF-like molecules in submaxillary gland, lacrimal gland, and kidney, frozen tissue pieces were homogenized in 4 volumes (wt/vol) of ice-cold phosphate-sucrose buffer (250 mM sucrose, 50 mM sodium phosphate, pH 7.4, supplemented with 2.5 µg/ml each of peptatin A, leupeptin, chymostatin, and antipain) with an Ultra-Turrax homogenizer for five 15-s bursts with 1-min intervals between bursts. The homogenate was centrifuged at 40,000 g for 20 min at 4°C. The resultant supernatant was retained and designated the soluble fraction and was the source of soluble EGF molecules either by RIA or Western blot. For RIA analysis, the amounts of EGF-like activities in the presence of irEGF. For Western blot analysis, the immunoprecipitates were collected by centrifugation for 15 min at 15,000 g at 4°C. Supernatants were aspirated, and pellets were monitored for [125]I-rEGF in the different fractions was estimated by comparison with a competition curve obtained with authentic rEGF.

Gel filtration analysis of the molecular mass form of membrane-associated EGF precursor molecules. Triton-extracted membrane-associated EGF-containing molecules in both kidney and lacrimal gland were characterized by gel filtration on 1.6 × 60-cm Sephacryl S-200 and 1.6 × 30-cm Bio-Gel P-10 columns. The columns were equilibrated at 4°C in lysis buffer B. Elution was performed in the same buffer at the flow rates of 13 ml/h for Sephacryl S-200 and 8 ml/h for the Bio-Gel P-10 column. Calibration of the Sephacryl S-200 column was performed with ferritin (440 kDa), chicken IgY (190 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and rat submaxillary gland EGF (5.3 kDa). Calibration of the Bio-Gel P-10 column was performed with chicken IgY (190 kDa), rat submaxillary gland EGF (5.3 kDa), and p-nitrophenol. An aliquot of the solubilized membrane proteins from each tissue was analyzed before (Sephacryl S-200) and/or after (Sephacryl S-200 and Bio-Gel P-10) trypsin hydrolysis as described above. In both cases, 1-ml fractions were collected for evaluation of their irEGF content by RIA either directly or after trypsin hydrolysis as indicated in RESULTS AND DISCUSSION.

Immunoprecipitation of membrane-associated EGF precursor molecules. For immunoprecipitation experiments, 1 ml of lacrimal gland lysates and 0.5 ml of kidney membrane lysates prepared in lysis buffer A were used. Immunoprecipitations were carried out overnight at 4°C under constant rocking in the presence of 8 µg of ppEGF1 in the absence or presence of 20 µg of peptide p437. Immune complexes were precipitated with 30 µl of protein A-Sepharose conjugate for 1 h at 4°C, and the immunoprecipitates were collected by centrifugation for 15 s at 10,000 g. Immunoprecipitates were then washed twice in lysis buffer A and finally in Tris-buffered saline buffer (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Immunoprecipitates were then analyzed for the presence of EGF precursor molecules either by RIA or Western blot. For RIA analysis, the washed protein A-Sepharose pellet was first resuspended in 100 µl of trypsin-containing buffer (100 µg trypsin/ml) and incubated for 1 h at 37°C. Protein A-Sepharose was pelleted by centrifugation, and the supernatant was tested for the presence of irEGF. For Western blot analysis, the immunoprecipitate was heated in SDS sample buffer for 5 min at 100°C. Solubilized proteins were separated by 7.5% SDS-PAGE, electrotransferred, and blotted overnight to nitrocellulose membrane (BA 85, Schleicher & Schuell, Dassel, Germany). Blots were then further processed and immunostained with affinity-purified anti-p437 antibody (ppEGF1; 0.8 µg/ml) and probed with a 1:1,000 dilution of goat anti-rabbit IgG.
antibody linked to horseradish peroxidase exactly as described previously (18). Blots were developed in ECL according to manufacturer recommendations and visualized by exposure to Amersham Hyperfilm-ECL.

RNA extraction and RT-PCR analysis. Total RNAs from brain, heart, liver, kidney, parotid glands, submaxillary glands, whole lacrimal glands, and lacrimal acinar cells from rats were prepared and subjected to reverse transcription as described previously (18). Oligonucleotide primers (22-mer) were used for amplification of the EGF precursor mRNA by PCR. The sense and antisense oligonucleotide sequences were obtained from a published cDNA sequence of the rat prepro-EGF (Ref. 34; GenBank no. M63585). They are listed in the 5’-to-3’ direction with the following coordinates: sense positions 3084–3105 (ATGTCTGCCAATGCTCAGAAGG) and antisense positions 3679–3700 (TAGGACCACAAACCAAGGTTGGG). After 30 cycles of amplification (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) performed in a Perkin-Elmer thermal cycler, amplified cDNAs were analyzed by electrophoresis on a 2% agarose gel in buffer containing 89 mM Tris, 89 mM boric acid, and 1 mM EDTA (pH 8) and identified by ethidium bromide staining as previously described (18). Nega- tive controls were carried out either with reverse transcription performed in the absence of RNA templates or with RNA incubated in the absence of RT. cDNAs were further transferred to Zeta-probe membranes (Bio-Rad) by capillary blotting overnight under high ionic strength (10× SSC buffer = 1.5 M sodium chloride and 0.15 M sodium citrate supplemented with 0.5% SDS wt/vol) and fixed covalently to the membrane by intense ultraviolet (UV) illumination.

Southern blot analysis of PCR amplification products. To verify the specificity of the amplification products, the hybridization of the Southern blots with a specific cDNA probe was performed. The probe used was the 0.4-kb fragment derived from cDNA clone pmEGF-26F12 of the mouse prepro-EGF (11) (American Type Culture Collection, ref. no. 37486). The probe was labeled by random priming with [α-32P]dCTP. The Northern blot was prehybridized, hybridized overnight in the presence of the labeled probe (3 × 10^6 cpm/ml), washed under high stringency, and autoradiographed by exposure to Amersham Hyperfilm using two intensifying screens at −80°C as described previously (18). To test for the integrity of the poly(A)⁺ RNA preparations from the different rat tissues, Northern blot analysis for β-actin mRNA was carried out with a 1150-bp fragment of the mouse β-actin cDNA in a manner similar to that described above.

RESULTS AND DISCUSSION

As stated in the introduction, the presence of EGF as well as its mRNA in lacrimal gland tissues and the presence of EGF in tears were recently documented. Moreover, it is now known that EGF mRNA encodes a high-molecular-mass precursor molecule from which EGF may be released by proteolytic cleavage. EGF may be present as a part of the extracellular portion of the transmembrane precursor (as in the kidney), or this precursor could be fully and intracellularly processed into EGF (as in the submaxillary gland). Until now, despite the demonstration of the presence of an EGF immunoreactivity in some lacrimal gland tissues, little attention has been given to the identification of the molecular form present in such tissues. The following experiments were thus performed with the rat exor- bital lacrimal gland to answer this question. We first used the highly sensitive technique of RT-PCR, in conjunction with specific oligonucleotide primers for the rEGF, to investigate the expression of the EGF mRNA in the lacrimal gland and for comparison with other rat tissues.

RT-PCR analysis of the tissue expression of EGF mRNA. As a control for the integrity of the RNA that serves as substrate for the RT-PCR analysis, agarose gel electrophoresis was performed, and the RNA was visualized with ethidium bromide under UV illumination (data not shown, but see Ref. 18). Equal amounts of highly preserved RNA appear to be present in the fractions. These RNAs were first reverse transcribed into cDNA and then amplified by PCR using specific sense and antisense primers deduced from the sequence of the rEGF precursor (34). Amplification primers were chosen to amplify a region of the precursor mRNA (nt. 3084–3700) that overlaps the sequence encoding the EGF molecule (nt. 3308–3466) (Fig. 1A). A single amplified product of the expected size (617 bp) was visible on an ethidium bromide-stained agarose gel (Fig. 1B). Strong signals were obtained with RNA from the submaxillary gland, lacrimal gland, and lacrimal acinar cells, as well as from kidney. Weaker signals were observed with both parotid and liver RNA, and no amplification signals were seen with brain and heart RNA. Moreover, no amplification products could be detected when the RNA template was omitted from the reaction (control) or when the amplification was performed with non-reverse-transcribed RNA (data not shown). These results show first that RNA templates were necessary to observe the amplification product and second that this product originates from mRNA rather than from potentially contaminating genomic DNA.
To verify the specificity of the 617-bp cDNA, the RT-PCR products were analyzed by the method of Southern blotting. As shown in Fig. 1A, we used a 32P-labeled mEGF cDNA probe (0.4-kb Pst I fragment of clone pmEGF-26F12) that was complementary to the 5' region of the amplified rEGF cDNA. Figure 1C shows that the 617-bp RT-PCR product hybridizes strongly with the probe with a signal intensity that appears to be proportional to that observed on the ethidium bromide-stained gel (Fig. 1B). Because high-stringency conditions were used to wash the hybridized membrane (0.1× SSC, 65°C), this demonstrates an important sequence homology between the probe and the 617-bp cDNA, strongly suggesting that it was the result of the RT-PCR amplification of the rEGF mRNA.

Because we were mainly interested in the demonstration of the presence of the EGF mRNA in the rat lacrimal gland, the specificity of the RT-PCR product obtained with the RNA from this tissue was further confirmed by restriction map analysis. The PCR products from both lacrimal and submaxillary glands, tissue that is known to contain the EGF mRNA, were extracted and digested with restriction enzymes that were predicted to provide specially sized fragments. The cleavage of the 617-bp cDNA from both tissues by Pst I, Sst I, and Hae III, alone or in combination, yielded shorter fragments of the predicted sizes (data not shown) and further confirmed the specificity of the amplification products obtained with both the submaxillary and lacrimal gland RNA.

Taken together, the results of the RT-PCR analysis indicate that the EGF mRNA was present in total RNA preparations from the expected rat tissues, i.e., submaxillary gland and kidney, but also from whole lacrimal gland and acinar cell preparations. Amplifications yielded weaker signals in both parotid gland and liver. The presence of EGF mRNA in the liver is rather controversial, since it was not detected in mouse liver by Northern blot (14, 33) but was clearly identified by RT-PCR in rat liver (24). RNA preparations from whole
rat brain and heart do not appear to contain any EGF transcript. These negative results were not the consequence of defective RNA preparations, since the preparations were previously shown to allow the amplification of the EGFR mRNA (18). The hypothesis of impaired reverse transcription reactions could also be ruled out, since PCR amplification reactions using the same cDNA preparation as a substrate demonstrated the presence of TGF-α mRNA in brain and of HB-EGF mRNA in both brain and heart RNA populations (data not shown). The absence of EGF transcripts in the rat brain is in agreement with the previous demonstration that the rat brain does not contain any detectable ir-EGF (32).

The demonstration of the presence of EGF mRNA by RT-PCR in the rat exorbital lacrimal gland confirms the results obtained with human (52) and mouse (14) lacrimal tissues. The intensity of the amplification signal in both lacrimal gland and acinar cell RNA preparations, compared with the submaxillary gland and kidney, suggests that they may contain relatively high amounts of EGF mRNA. However, because of the high sensitivity of the technique, it is known that quantification of a specific transcript as well as comparison from tissue to tissue by RT-PCR is rather difficult. Thus, to have a better estimation of the relative amounts of EGF mRNA and to determine the size of the transcript, we decided to analyze the poly(A)^+ RNA from several rat tissues, i.e., submaxillary and lacrimal glands, lacrimal acinar cells, liver, brain, and kidney, by Northern blot.

Northern blot analysis of poly(A)^+ RNA. The 617-bp PCR product was used as a probe after ^32P labeling by random priming. Northern blots were hybridized with the labeled probe and washed under high-stringency conditions as described in MATERIALS AND METHODS. Hybridization revealed one specific 5-kb transcript in our control tissues, i.e., in submaxillary gland and kidney as well as in lacrimal and liver preparations (Fig. 2A). The size of this transcript is close to that of the prepro-EGF mRNA identified by Northern blot in mouse kidney and submaxillary gland (14) and only slightly longer than the rat prepro-EGF cDNA (4801 bp) cloned from rat kidney (34). This Northern blot analysis clearly shows that the lacrimal gland contains significant amounts of prepro-EGF transcript. The tissue content could be estimated to be about one-tenth of that present in both submaxillary gland and kidney. Contrary to the results of Kasayama et al. (14) obtained with mouse tissues, we did not find any difference in the size of the transcript between the lacrimal gland and kidney.

According to the RT-PCR analysis, the prepro-EGF transcript was also clearly detected, although at a barely detectable level, in the liver, and no transcript could be detected in the poly(A)^+ RNA preparation from the rat brain. In each tissue, the 2.1-kb mRNA for the structural protein β-actin shows an intense and nearly equal hybridization signal (Fig. 2B). This confirms the integrity of the different mRNA preparations and indicates that the variations in EGF mRNA signals observed between the different rat organs are not the consequence of either mRNA degradation or important variations in the mRNA loading.

Taken together, these results indicate that the RT-PCR amplification product observed with lacrimal gland and lacrimal acinar cell preparations presumably reflects the presence of mRNA encoding prepro-EGF. Thus the following experiments were designed to determine whether translation of this mRNA in the rat lacrimal gland results in the presence of the entire membrane-associated EGF precursor protein or of a fully processed and soluble low-molecular-mass EGF molecule.

Assay of EGF-related molecules in soluble fractions from submaxillary gland, lacrimal gland, and kidney by RRA. The next experiments were designed to deter-
mine the presence of EGF or EGF-related molecules in the rat lacrimal gland. We first looked for the presence of soluble EGFR binding proteins in the soluble fractions from control tissues, i.e., submaxillary gland and kidney, and compared the results with those of the lacrimal gland. In these experiments, serial dilutions of the soluble fractions from the different tissues were tested for their ability to compete with $^{125}$I-rEGF for binding to the rat liver EGFR. As can be seen in Fig. 3, all three soluble fractions compete with $^{125}$I-rEGF for binding to the EGFR and show displacement curves parallel to that of purified rEGF. Comparison of these curves with that of standard EGF allowed an estimation of the amounts of EGF-like molecules present in the different tissues. As could be predicted, submaxillary glands contain the highest amount of soluble EGF-like activity (5,700 pg/mg tissue), whereas kidney (340 pg/mg tissue) and lacrimal glands (200 pg/mg tissue) contain lower and comparable activities. Taking into account our knowledge that EGF is not the only EGFR binding molecule, these results only indicate that the lacrimal gland contains soluble EGF-related activities. EGF might be only one of these activities, since TGF-$\alpha$ has been demonstrated to be present in the rat lacrimal gland (47) and since we have recently identified both TGF-$\alpha$ and HB-EGF transcripts in addition to EGF in the same tissue by RT-PCR (data not shown). Thus the determination of the specific contribution of EGF in this EGFR binding activity requires development of a specific and sensitive RIA for rEGF.

RIA for rEGF. Polyclonal antibodies were produced by immunizing rabbits against a purified and native preparation of rat submaxillary gland EGF (rEGF). The IgG fraction of the antisera containing the highest titer was purified through protein A-Sepharose chromatography as described in MATERIALS AND METHODS. An RIA was thus developed using $^{125}$I-rEGF and the anti-rEGF antiserum rEGF2. As shown in Fig. 4, for an antibody dilution of 1:5,000, rEGF inhibits >95% of $^{125}$I-rEGF binding, with a half-maximal inhibition at an rEGF concentration between 0.1 and 0.2 nM. These experimental conditions allowed the detection of 60 pg/assay of rEGF. This antibody is highly selective for rEGF. Although mEGF has very high sequence homology (79%) with rEGF, 100 times more of this growth factor is required for comparable displacement. Moreover, mEGF displaced $^{125}$I-rEGF in a nonparallel fashion compared with rEGF, indicating the nonidentity of the antigenic determinants. The antibody rEGF2 only weakly recognized hEGF, despite its 67% homology with rEGF, and did not recognize rat TGF-$\alpha$ (35% homology) at concentrations as high as 100 nM. Taken together, these results show that this antibody directed against native rEGF clearly cross-reacts with closely related molecules as a function of their sequence homology with rEGF. These results are in striking opposition to those obtained with RRA experiments in which we observed that all these molecules compete with equal potency when binding to the EGFR (data not shown).
Because TGF-\(\alpha\), which has been shown to be present in the rat lacrimal gland (47), shows the highest homology with EGF in the family of EGFR binding growth factors, it is clear that the rEGF\(_2\) antibody that did not bind TGF-\(\alpha\) can be used to specifically identify EGF in this tissue. The other advantage of RIA vs. RRA is that it allows the measurement of EGF or EGF-containing molecules in both soluble fractions and membrane preparations solubilized in the presence of detergents. This was the subject of the following experiments.

Assay of EGF in soluble fractions from submaxillary gland, lacrimal gland, and kidney by RIA. To measure the level of irEGF in the soluble fractions from the different tissues, serial dilutions were assessed for their ability to compete with \(^{125}\text{I}-\text{rEGF}\) in binding to the rEGF\(_2\) antibody. As was proposed by Schaudies et al. (36), to detect the presence of EGF-containing molecules (precursor) that may have antigenic properties different from those of mature rEGF, samples of lacrimal gland and kidney were tested both before and after tryptic digestion as described in MATERIALS AND METHODS. Mature EGF is insensitive to trypsin (38), and tryptic digestion of EGF precursor molecules releases EGF in a form that is both immunologically and biologically indistinguishable from mature EGF (Ref. 36 and see Fig. 7).

As is shown in Fig. 5, soluble extracts of submaxillary gland fully compete with \(^{125}\text{I}-\text{rEGF}\) in binding to the rEGF\(_2\) antibody. This immunoreactive material competes with \(^{125}\text{I}-\text{rEGF}\) in a manner parallel to purified EGF. The displacement curve is unaffected by previous tryptic digestion of the sample (data not shown), suggesting that it is composed of mature soluble EGF. Calculation of the amount of EGF in this fraction gave a value of 5,200 pg/mg of tissue. This value is only slightly less than the one determined by the RRA experiments (5,700 pg/mg). As could be predicted, this indicates that EGF makes up most of the soluble EGFR binding activity in the submaxillary gland.

The results obtained with soluble fractions from both the kidney and the lacrimal gland are completely different. Although very significant amounts of soluble EGFR binding activities could be detected by RRA in these tissues, we found, surprisingly, that they contain either very low (kidney) or undetectable (lacrimal) levels of immunoreactive material. However, tryptic digestion of the kidney fraction resulted in an important increase of immunologically active material (from 2.9 to 23 pg/mg of tissue) that demonstrates a displacement curve parallel to that of purified rEGF. In the same conditions, the lacrimal gland fraction still remains negative.

Taken together, these results indicate that soluble rat kidney EGF is mainly in the form of EGFR-containing trypsin-sensitive precursor molecules. This result is different from that of Schaudies and Johnson (37), who found that EGF was mainly present as mature EGF in this fraction. We homogenized the different tissues in the presence of protease inhibitors, whereas Schaudies and Johnson (37) did not, and this may explain the discrepancy. Thus extensive proteolysis of soluble EGF precursors may have occurred in the course of homogenization, leading to the production of mature EGF, whereas this effect may have been very limited in our experiments. The detection of soluble EGF precursor molecules in the kidney is consistent with the demonstration that these types of molecules are present in the urine of different species (13, 15–17, 25).

Surprisingly, we were unable to detect either mature EGF or any EGF precursor in the soluble fraction from the rat lacrimal gland. Taking into account the sensitivity of our RIA, our results mean that the level of irEGF in the rat lacrimal gland soluble fraction was <0.3 pg/mg of tissue. This value is far below the amount of EGFR binding activity present in the same fraction (200 pg/mg tissue) as measured by RRA and clearly indicates that the EGF-like activity of the rat lacrimal gland soluble fraction is not made up of EGF or EGF precursor molecules. TGF-\(\alpha\) and/or HB-EGF may be part of this activity, since TGF-\(\alpha\) has been demonstrated to be present in the rat lacrimal gland (47) and since we have recently identified both TGF-\(\alpha\) and HB-EGF transcripts in addition to EGF in the same tissue by RT-PCR (data not shown). These observations are discrepant with previously published results showing the presence of soluble mature EGF in both mouse
METHODS. Serial dilutions (up to 1:100) were performed in site to what was observed with the untreated samples, samples to compete for binding to the antibody. Opposite results in a dramatic increase in the ability of the trypsin hydrolysis of both lacrimal gland and kidney Triton X-100 extracts. As can be seen in Fig. 6, the soluble fractions, tryptic digestion of the sample the immunoreactive materials. As discussed above for the soluble fractions, trypsin digestion of the sample was used to test for the presence of EGF precursor in the Triton X-100 extracts. As can be seen in Fig. 6, trypsin hydrolysis of both lacrimal gland and kidney results in a dramatic increase in the ability of the samples to compete for binding to the antibody. Opposite to what was observed with the untreated samples, the trypsin-treated samples generated displacement curves that were parallel to the standard curve, thus suggesting the generation of mature irEGF from precursor molecules. After trypsin hydrolysis, the level of irEGF detected rose from 1.6 to 25.7 pg/mg of tissue in the lacrimal gland and from 9.5 to 110 pg/mg of tissue in the kidney. As stated above, it is now known that the rat kidney contains membrane-associated EGF precursor molecules (37). Thus, by analogy with the kidney, the presence of the trypsin-sensitive EGF-containing molecules in the Triton-solubilized membrane fraction from the rat lacrimal gland strongly suggests the existence of membrane-associated EGF precursor molecules in this tissue.

Characterization of the detergent-extracted membrane-associated EGF precursor molecules by size exclusion chromatography. The following experiments were performed to determine the size of the EGF-containing molecules that generate immunoreactive EGF on trypsin treatment as well as the size of the material released by trypsin. Triton-solubilized membrane fractions from both the kidney (Fig. 7, A and C) and the lacrimal gland (Fig. 7, B and D) were analyzed by size exclusion chromatography either on Sephacryl S-200 (Fig. 7, A and B) or Bio-Gel P-10 (Fig. 7, C and D) as described in MATERIALS AND METHODS.

At first, undigested materials were analyzed through Sephacryl S-200 chromatography (separation range 5–250 kDa), and aliquots of each eluate fraction were tested by RIA after tryptic digestion. Because the column was calibrated with proteins of known molecular masses, the results show that the trypsin-sensitive EGF-containing material from the kidney (Fig. 7A) and lacrimal gland (Fig. 7B) eluted as proteins of high apparent mass. In both cases, the maximal EGF-releasing activity was associated with fractions corresponding to apparent molecular masses close to 160–180 kDa. However, the peak of activity generated by the kidney fractions appears to be more heterogeneous than that generated by the lacrimal fractions. This may indicate a more marked heterogeneity in the EGF-generating material present in kidney membranes.

Sephacryl S-200 analysis of the Triton-solubilized membrane fractions after trypsin treatment resulted in a shift of the peak of activity for both the kidney (Fig. 7A) and the lacrimal gland (Fig. 7B). In both cases, all the EGF immunoreactivity was now contained in a single and sharp peak of low molecular mass that coeluted with the purified rat submaxillary gland EGF. However, because this trypsin-generated material as well as standard EGF eluted with the total volume of this type of column, it was not possible to accurately estimate its molecular mass. Thus the trypsin-treated samples were further analyzed through Bio-Gel P-10 chromatography that permits the separation of proteins of molecular masses between 1 and 20 kDa. As shown for both kidney (Fig. 7C) and lacrimal gland (Fig. 7D), the trypsin-generated EGF immunoreactivity eluted as a single peak of activity that still coeluted with purified rEGF.
Taken together, these results show that the EGF immunoreactivity generated by trypsin hydrolysis of the Triton-solubilized membrane fraction from both kidney and lacrimal gland was indistinguishable from purified rEGF by size exclusion chromatography. Because the trypsin-sensitive EGF-releasing material is associated with high-molecular-mass proteins, the results strongly suggest that these proteins are membrane-associated EGF precursor molecules.

Immunoprecipitation and Western blot analysis of the detergent-extracted membrane-associated EGF precursor molecules. The above size exclusion chromatographic analysis of the detergent-solubilized membrane-associated EGF immunoreactivity only provided rough estimates of the EGF precursor(s) molecular mass(es). So we tried to obtain more accurate values by using an antibody (ppEGF1) raised against a synthetic peptide (p437) that corresponds to a sequence of the rat prepro-EGF that is predicted to be located in its intracellular juxtamembrane domain. Because of the location of this antigenic determinant, ppEGF1 antibody is postulated to identify only membrane-associated precursor molecules.

Triton-solubilized membrane fractions from the kidney and lacrimal gland were first immunoprecipitated in the absence or presence of the antibody ppEGF1. The specificity of the immunoprecipitation was assessed by performing the incubation with ppEGF1 in the absence or presence of a saturating concentration of the peptide p437, as described in MATERIALS AND METHODS. Immunoprecipitates were subsequently analyzed by Western blot using the ppEGF1 antibody. As shown in Fig. 8, these proteins appear to contain only one specific immunoreactive protein with an apparent molecular mass of 152 kDa. This protein is also present in the immunoprecipitate from the kidney membrane, together with three other proteins with apparent molecular masses of 115, 97, and 75 kDa. A final demonstration that these are EGF precursor molecules would necessitate the demonstration that they also contain the sequence of EGF. Unfortunately, our anti-rat EGF antibody (rEGF2) and most of the anti-native EGF antibodies do not efficiently recognize denatured EGF and cannot be used in Western blot analysis. However, indirect evidence that EGF is present in both kidney and lacrimal gland ppEGF2 immunoprecipitates has been obtained in parallel experiments. The protein A-Sepharose-recovered ppEGF2 immunoprecipitates from both tissues were first incubated in the presence of trypsin. The incubation media (protein A-Sepharose supernatants) were subsequently analyzed by RIA for the presence of EGF immunoreactivity using the rEGF2 antibody. Both kidney and lacrimal gland immunoprecipitates were shown to contain trypsin-released immunoreactive EGF. The specificity of these results was
METHODS. Blots were developed with enhanced chemiluminescence (ECL) and visualized by exposure to Amersham Hyperfilm-ECL. Molecular mass markers are indicated at left, in kDa. Immunoprecipitated proteins are indicated at right.

Further confirmed by showing that the addition of p437 during the immunoprecipitation phase completely precluded the detection of this EGF immunoreactivity (data not shown).

The presence of the 152-kDa protein in the immunoprecipitates from both the lacrimal gland and kidney suggests that it may be the full-length membrane-associated EGF precursor. This molecular mass is higher than the one predicted and calculated from the primary sequence of the molecule (34). Because the sequence of the rEGF precursor contains six potential sites for N-glycosylation in the extracellular portion of the molecule, this suggests that, like its human (23) and mouse (2) counterparts, it may be glycosylated.

Our results also point out an important difference between the lacrimal gland and kidney. Indeed, at steady state, only the full-length membrane-associated EGF precursor appears to be present in the lacrimal gland, whereas in the kidney the presence of at least three other proteins of lower molecular mass (115, 97, and 75 kDa) may point to the partial processing of the precursor in its NH2-terminal, extracellular part of the molecule. Thus cleavage of these partially processed membrane precursors at the COOH-terminal (distal) end of EGF that releases soluble EGF-containing molecules could easily explain the presence of the soluble trypsin-sensitive EGF-containing molecules in this tissue (see discussion of Fig. 5 above). However, the possibility that some proteolytic cleavage in the NH2-terminal proreregion of the kidney EGF precursor may have occurred during tissue processing in spite of the presence of the cocktail of protease inhibitors (see MATERIALS AND METHODS) cannot be ruled out. Indeed, it was recently shown that rat kidney membrane fractions contain protease activities that were able to sequentially and fully process the membrane-associated EGF precursor into soluble mature (6-kDa) EGF (12, 13). Meanwhile, these protease inhibitors may have efficiently prevented the complete processing of both membrane-associated and soluble EGF precursors, since, contrary to the findings of Schaudies and Johnson (37), we observed that soluble EGF appears to be mainly present in the form of trypsin-sensitive EGF-containing molecules. In the lacrimal gland, the observation of only one form of high-molecular-mass (152 kDa) membrane-associated pro-EGF suggests that the partial processing of the precursor in its NH2-terminal proreregion does not occur. This lack of significant precursor processing may be explained by a lower level or a complete absence of "noninhibitable" protease activities. Nevertheless, we cannot completely exclude the possibility that the amount of partially processed pro-EGF was below the detection limit of the Western blot analysis.

Up to now, it is not known whether the partial cleavage of pro-EGF in its proximal extracellular portion, i.e., cleavage of the precursor molecule at the distal and juxtamembrane sites, is involved in the regulation of EGF secretion. From both in vivo and in vitro studies, it appears that the membrane precursors of the different members of the EGF family may undergo differential processing before the release of the soluble forms of the growth factors. The transmembrane TGF-α precursor molecule is rapidly cleaved in its NH2-terminal proreregion, releasing all the glycosylated part of the molecule and leaving TGF-α membrane anchored. The release of mature soluble TGF-α by the cleavage of the resulting, lower-molecular-mass, TGF-α-containing, membrane-anchored form is a highly regulated process (28, 29). In the case of amphiregulin, cleavage of the proreregion led to the generation of a predominant 43-kDa soluble form that may retain the full-length NH2-terminal proreregion (4). The release of this amphiregulin ectodomain was also shown to be regulated (4).

If we look at EGF, it is clear that tissues such as submaxillary glands from rodents fully and intracellularly process pro-EGF into mature 6-kDa EGF that is secreted through regulated exocytosis (6, 31, 33, 39). However, as suggested from in vivo studies performed with urine and milk (16, 17, 21, 30), the predominant EGF species released from most epithelial cells appears to be a high-molecular-mass, 160-kDa form. Moreover, both NIH/3T3 cells (23) and Madin-Darby canine kidney cells (9) stably transfected with the human pro-EGF cDNA have demonstrated that membrane-associated pro-EGF is only present as a single high-molecular-mass precursor that is proteolytically cleaved to release a high-molecular-mass, soluble 160-kDa EGF form, without any evidence for the generation of mature 6-kDa EGF. From these observations, it seems
that our results with the rat lacrimal gland greatly resemble those obtained with in vitro transfected cells. If the comparison can be further extended, we may suggest that the primary product secreted by this gland would also be high-molecular-mass EGF. This high-molecular-mass (trypsin-sensitive) soluble pro-EGF, which would be present in the extracellular tissue medium, was not observed in the course of our study. This may be the result of a low rate of precursor cleavage at the distal site and/or rapid wash-out of the extracellular tissue medium before tissue processing. Both of these phenomena would contribute to the lowering of the steady-state level of soluble immunoreactive EGF below the RIA threshold. It is clear that further studies using immunoprecipitation experiments after metabolic labeling of the tissue as well as kinetic analysis of the secretory product(s) are needed to test this hypothesis. However, we can propose a model for the action of this secreted pro-EGF. As stated in the introduction, tear EGF at the ocular surface has been reported to be present as the 6-kDa growth factor (27, 45, 50) that is suggested to be involved in at least in the regulation of corneal wound healing (5, 35, 51). We recently observed that plasmin, a serine protease that is also present in tears at elevated levels in some corneal diseases (7, 43, 44), was able to fully process the membrane-anchored pro-EGF into the mature soluble 6-kDa EGF (20a). So we propose that soluble high-molecular-mass pro-EGF present in the aqueous flow coming from the lacrimal gland could be matured into the well-known 6-kDa growth factor only when reaching the ocular surface. Under these conditions, a detectable amount of soluble pro-EGF could be present in tears, a question that has never been addressed to our knowledge. This does not mean that the only role of the pro-EGF soluble form would be to produce the 6-kDa growth factor. Indeed, soluble pro-EGF forms have been reported to bind and activate the EGF receptor (2, 22, 21, 29, 30, 36, 37), but the question remains as to whether it is their only function and, if so, whether they induce exactly the same cellular response.

In conclusion, this study demonstrates the transcription of the EGF precursor gene in the rat exorbital lacrimal gland. The soluble, mature, low-molecular-mass form of EGF was undetectable in this tissue. However, as in the kidney, EGF is present in the form of its membrane-associated high-molecular-mass (152 kDa) precursor. Because we have detected relatively high amounts of EGF binding activity in the soluble fraction from the rat lacrimal gland, this could indicate that other growth factors of the EGF family are present in this tissue. In these conditions, the EGF-like immunoreactivity previously detected and localized in the duct cells of the rat exorbital lacrimal gland (48, 50) could represent either some immunoreactivity cross-reacting with one of these soluble growth factors or the detection of the EGF-containing membrane-associated precursor protein.

Location of the site(s) of synthesis of the EGF precursor mRNA and protein in the rat lacrimal gland, by both in situ hybridization and immunohistochemistry, and investigation of the way(s) by which EGF could be released from its precursor into tears are now required.

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