Synthesis and Storage of Epithelial-Epidermal Growth Factor in Submaxillary Gland

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SUMMARY

Epithelial-epidermal growth factor (EGF) is a purified protein derived from mouse submaxillary gland that appears to function as a hormone to induce the growth of epidermis and of normal and neoplastic mammary cells. Immunofluorescent staining demonstrated the presence of this factor in specific tubular cells of the submaxillary gland, although it was not detected in any other mouse tissues. EGF was also detected in the rat and rabbit by its immunological cross-reactivity with the mouse antigen and by its biological activity on mouse mammary gland. However, it was not detectable by these criteria in the submaxillary glands of several other species including the human. Organ cultures of mouse submaxillary gland incorporated $^{14}$C-labeled amino acids into protein that was shown to be identical to authentic EGF by polyacrylamide gel electrophoresis after purification by specific immunoabsorption. This result indicates that EGF is synthesized by mouse submaxillary cells and thus suggests that elaboration of EGF may be a specific function of these cells.

INTRODUCTION

EGF is a polypeptide which has been previously isolated in pure form from the submaxillary glands of male mice. Although the biological functions of EGF have not been completely elucidated, it has been demonstrated that EGF stimulates epidermal growth in several species. The observations that it stimulates cell proliferation in mammary epithelial cells and mammary carcinoma cells in organ cultures at concentrations similar to those of polypeptide hormones in plasma have suggested that EGF may function as a growth hormone for several types of epithelial cells. Since the submaxillary gland contains numerous cell types, it was of interest to determine the cell of origin of the EGF. The submaxillary gland is generally considered to be primarily an exocrine organ with the ability to concentrate various substances. The presence of this biologically active polypeptide in the submaxillary cells, therefore, raised the additional question as to whether it is a biosynthetic product of this gland or whether it is formed in some other site and merely stored in enriched concentration in this salivary gland. A monospecific anti-EGF antibody preparation has been used to provide direct evidence relating to these questions and to determine the tissue specificity of EGF. Finally, the species distribution of EGF-like polypeptide has been studied by immunological cross-reactivity and by assays for biological activity.

MATERIALS AND METHODS

Preparation of EGF. Highly purified EGF was prepared from the submaxillary glands of male mice as previously described. The preparation contained a single component on polyacrylamide gel electrophoresis at acrylamide concentrations between 7 and 20% and at pH 8.60 or 9.56, and it behaved as a single peak in the analytical ultracentrifuge. Submaxillary glands removed from other male animals at the abattoir or from human male subjects at surgery were rapidly frozen on Dry Ice. The tissues were subjected to the same purification steps used for the preparation of EGF from mouse submaxillary glands through the heating step.

Preparation of Anti-EGF. Anti-EGF $\gamma$-globulin was prepared by inoculating rabbits in the toe pads with purified EGF in Freund's adjuvant. $\gamma$-Globulin was prepared from the plasma by precipitation with 50% ammonium sulfate and was labeled by incubation with fluorescein isothiocyanate at 4° for 24 hr. The fluorescein not bound to protein was removed by dialysis and by gel filtration through Sephadex G-25.

Immunofluorescent Staining. Detection of antigen in tissues was performed by a modification of the direct staining method of Coons. Frozen tissue was sectioned at 5 $\mu$, and the stained sections were examined for fluorescence as previously described.

Organ Culture Techniques. Explants of C3H/HeJ mouse midpregnancy mammary gland were incubated on sterile Medium 199 for 24 hr as previously described. They were then exposed to medium containing thymidine-3H (Schwarz BioResearch, Inc., Orangeburg, N. J., methyl labeled; specific activity, 8.0 Ci/mmmole) at a concentration of 0.2 $\mu$Ci/ml. DNA was isolated and counted with an accuracy of ±1% as previously described. Submaxillary gland explants (0.2 mg) representing all the tissue derived from 1 or 2 mice were floated on siliconized lens paper rafts on sterile minimal Eagle's medium (Microbiological Associates, Bethesda, Md.) containing 20 mg NaHCO$_3$/ml. The incubation was continued for 4 hr at 37° in an atmosphere of 5% carbon dioxide and air.
Isolation of EGF-$^{14}$C. Salivary explants were exposed for 4 hr to medium containing a mixture of $^{14}$C-labeled amino acids (Schwarz reconstituted protein hydrolysate) at a concentration of 10 $\mu$Ci/ml or lysine-$^{14}$C (40 $\mu$Ci/ml). The tissue (100 to 200 mg) was then homogenized in 0.8 ml 0.15 M sodium chloride containing 0.02 M Tris-HCl, pH 7.4, and the homogenate was centrifuged at 17,000 $\times$ g for 20 min. The supernatant was then heated at 90° for 5 min, denatured protein was removed by centrifugation, and the EGF was purified from the supernatant by immunoadsorption. For this purpose, monospecific anti-EGF globulins were complexed with bromacetyl cellulose by the method of Jagendorf et al. (6). A suspension of 10 mg of the cellulose-globulin complex was incubated with the tissue supernatant in a final volume of 1.0 ml for 20 min at 27°. The cellulose-protein complex was removed by centrifugation and washed 4 times with 0.15 M sodium chloride. Absorbed antigen was then removed from the antibody by incubation in 0.2 N HCl for 10 min at 27°. The supernatant containing the dissociated antigen was neutralized and dialyzed against water. Purified EGF “carrier” (75 $\mu$g) was added, and the solution was then lyophilized. The dry protein was dissolved in 150 $\mu$l of 6 M urea-10% sucrose and subjected to electrophoresis in polyacrylamide gels at 30°. The lower gel was 10 to 20% acrylamide with ethylene diacrylate as cross-linker, and was buffered at pH 8.60 or 9.56 with Tris-HCl. The upper gel was 5% acrylamide with bisacrylamide cross-linker and was buffered at pH 6.86 with trisphosphoric acid. Both gels contained 6 M urea, and the reservoir buffers were those of Jovin et al. (7). After electrophoresis at 10 ma/tube, the gels were cooled and scanned at 280 m$\mu$ in the Gilford recording spectrophotometer with a linear transport device and a slit width of 0.08 m$\mu$. Uniform gel sections 1.0 mm thick were dissolved in 0.2 N NaOH and then were neutralized and counted in toluene phosphor containing Triton X-100.

RESULTS

The anti-EGF antiserum formed a single precipitin band on immunodiffusion against mouse EGF, as shown in Fig. 1a. Precipitin bands formed at dilutions of antiserum from 1:1 to 1:128 and at mouse EGF concentrations of 1 and 5 mg/ml. The anti-EGF was shown to be monospecific for EGF by immunoelectrophoresis (Fig. 1b).

The fluorescent antibody technique was used to localize the EGF in the salivary tissue. Fig. 2a shows a section of a male mouse submaxillary gland stained for EGF by the fluorescein-labeled anti-EGF $\gamma$-globulin preparation. The tubular cells were uniformly and brilliantly fluorescent, while the acinar cells and connective tissue elements showed no fluorescence above background. The tubular systems in the submaxillary gland of the adult female that stained with fluorescent antibody were of rudimentary size (Fig. 2b) in comparison to those of the male. However, 4 days after a single injection of testosterone cypionate (5 mg in oil s.c.), the submaxillary gland of the female mouse (Fig. 2c) contained a well-developed tubular system of cells which stained for EGF in a manner similar to the male gland. Treatment of these tissue sections with anti-EGF $\gamma$-globulin that was not labeled with fluorescein prevented the binding of fluorescein-labeled anti-EGF to these tissue sites, although $\gamma$-globulin derived from rabbits not immunized to EGF did not interfere with the fluorescent staining reaction. No fluorescence was bound

Chart 1. A, polyacrylamide electrophoretogram of mouse submaxillary gland protein labeled with mixed $^{14}$C-labeled amino acids and prepared as described in the text. EGF carrier protein was added prior to electrophoresis. Absorbance at 280 m$\mu$ (smooth tracing) is compared with radioactivity profile (histogram). One-mm gel sections were dissolved and counted for a period long enough to give a counting accuracy of $\pm$1%, and the background of 30 cpm was subtracted. B, polyacrylamide electrophoretogram of proteins derived from male mouse submaxillary gland exposed to lysine-$^{14}$C. Proteins were prepared, subjected to coelectrophoresis with authentic EGF, and counted as in Chart 1A, polyacrylamide electrophoretogram absorbance pattern at 280 m$\mu$ of authentic EGF. Protein 75 $\mu$g, was subjected to electrophoresis under the same conditions as in Chart 1A and B.
Table 1

Detection of EGF-like materials in submaxillary glands of various species

Each gland was rapidly frozen, and sections were stained with fluorescein-labeled anti-EGF. The frozen glands from each species were combined, homogenized, and processed in 2 batches by the procedure for isolation of EGF (see "Materials and Methods"). Submaxillary gland protein preparations were lyophilized and added to the incubation medium at the concentrations indicated. The rate of DNA synthesis represents the mean of closely agreeing replicate determinations.

<table>
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<th>Source</th>
<th>No. of specimens</th>
<th>Immunofluorescent staining</th>
<th>Protein concentration (µg/ml)</th>
<th>cpm/mg tissue/4 hr</th>
<th>% increase</th>
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specifically when these tissues were treated with fluorescein-labeled γ-globulin from rabbits not immunized to EGF. Serial sections of all other tissues of the mouse were similarly studied. EGF was not detectable by this technique in any other tissue, including parotid and sublingual salivary glands, lacrimal glands, and pancreas.

For determination of whether the immunoreactive material in the tubular cells was actually synthesized there or was merely stored by these cells, explants of male mouse submaxillary gland were allowed to incorporate 14C-labeled amino acids into protein in organ culture, and EGF was isolated from homogenates of the explants by immunoabsorption to anti-EGF.

Chart 1A is an electrophoretogram of the final salivary protein preparation obtained by linear absorbance scanning at 280 m

The large peak with the same electrophoretic mobility as EGF (Chart 1C) was observed in addition to 2 minor peaks and the origin and front (bromphenol blue) materials. A portion of the absorbance may represent bromphenol blue marker dye which is absorbed to the purified protein. Chart 1A shows that the major peak of protein 14C corresponds exactly to the EGF peak, and that most of the radioactivity is in this peak. This peak of radioactivity also had the same Rf as EGF identified by staining with Amido black. For a further test of the identity of the isotopically labeled protein in the EGF peak, lysine 14C-labeled salivary proteins prepared as above were subjected to electrophoresis, as shown in Chart 1B. EGF is known not to contain any lysine residues (11), in marked contrast to most animal proteins. A lysine 14C-labeled protein peak corresponding to EGF was not detected, although this amino acid was incorporated into other, minor protein peaks. The fact that other minor proteins contaminated the immunoabsorbed EGF does not indicate that the anti-EGF may react nonspecifically with other salivary proteins or that the immunofluorescent staining may be nonspecific. Nonspecific adsorption of protein by bromacetyl cellulose has been previously observed. (6). The results shown in Chart 1, A to C, are representative of 3 such complete studies.

To determine whether EGF-like material could be detected in the submaxillary glands of other species, we studied submaxillary tissues by immunofluorescent staining and assayed extracts for EGF-like activity. Previous studies demonstrated that the addition of EGF to the synthetic medium of mouse mammary organ cultures stimulated the rate of DNA synthesis in the mammary epithelial cells. EGF concentrations at least as low as 0.005 µg/ml produced a marked stimulation in the rate of DNA synthesis. Table 1 shows the results of studies on submaxillary glands of various species. In the mouse, rat, and rabbit, positive tissue staining of submaxillary tubular epithelial cells was observed. This positive tissue staining for EGF was associated with EGF-like biological activity in the salivary protein preparations from each species. However, the presence of EGF could not be detected in the submaxillary glands from other species tested by these immunological or biological tests.

DISCUSSION

These experiments indicate that mouse submaxillary gland incorporates amino acids into EGF polypeptide. The isotopically labeled protein shares a number of characteristic properties with authentic EGF: (a) immunoreactivity with monospecific anti-EGF γ-globulin; (b) heat stability; (c) electrophoretic mobility identical to EGF in 10 to 20% polyacrylamide gels containing 6 M urea; (d) failure to incorporate lysine 14C, an amino acid present in most animal...
proteins but absent from EGF. EGF is thus presumably synthesized in the tubular cells of the submaxillary gland, the only salivary cells found to contain the factor by immunofluorescent staining. The fact that no other tissues of the mouse were found to contain the factor further supports the concept that EGF is formed by the submaxillary tubular cells and that these cells do not merely store EGF after removal of the preformed protein from the plasma.

These results also extend the observations of several previous studies with EGF. Although the earlier and less sensitive bioassays of EGF, based upon its acceleration of eye opening in newborn mice, failed to detect the presence of this factor in the female submaxillary gland, the present studies clearly demonstrate its presence in the female tissue. The apparently lower concentration of the EGF in the female gland results from the smaller number of tubular cells present. These cells exhibit sexual dimorphism (8) and can be induced to proliferate and synthesize larger amounts of EGF by injections of testosterone. This documentation of EGF in the female animal further supports its potential role as a potent stimulator of cell proliferation in mammary gland and in mammary carcinomas (12, 13). The results indicate that EGF in the mouse is formed uniquely by the submaxillary gland and suggest that elaboration of EGF may be a specific function of these cells.

The failure to detect EGF-like material in the several other species tested may relate to several possible conditions: (a) these species may form EGF, but only in other tissues; (b) submaxillary EGF may exist in these species, but its physical properties are so different from mouse EGF that other purification procedures are required for its isolation; (c) submaxillary EGF in these species does not cross-react immunologically with anti-mouse EGF, and mouse mammary gland exhibits species specificity according to which only the mouse, rat, and rabbit materials are biologically active; (d) EGF is limited in its species distribution, and the negative results indicate its absence in the species tested. Further studies will be required to distinguish between these possibilities.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. a, immunoprecipitation of anti-mouse EGF antiserum (center well) with mouse EGF (0.125 μg/ml; Wells 1, 4, and 5). Well 2, mouse placental protein preparation, 1 mg/ml; Well 3, mouse parotid preparation, 1 mg/ml; Well 6, Rhesus monkey submaxillary protein preparation, 1 mg/ml. b, immunoelectrophoretic patterns of EGF-anti-EGF reaction. EGF (1 mg/ml) was placed in wells and subjected to electrophoresis (10). Antiserum (troughs) shows monospecific precipitation (9).

Fig. 2. Fluorescence photomicrographs of sections of mouse submaxillary gland stained with fluorescein-labeled anti-EGF γ-globulin. x 250. Note fluorescence of tubular cells. a, adult male; b, adult female; c, gland of an adult female treated 4 days previously with testosterone cypionate.
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