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Biochemistry

The biosynthetic precursor of epidermal growth factor and the mechanism of its processing

(arginyl esteropeptidase/protein processing/submaxillary gland/epidermal growth factor binding protein/high molecular weight epidermal growth factor)

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ABSTRACT The biosynthesis of epidermal growth factor (EGF) was studied in mouse submaxillary glands incubated with L-[^{35}S]cystine. EGF and EGF-like proteins were isolated from the gland homogenates by immunoprecipitation with anti-EGF antiserum. The major species appearing after short labeling periods is significantly larger (M_r, 9000) than EGF. The label in the M_r 9000 species plateaus after 1 hr whereas that in EGF continuously increases. When glands are chased with unlabeled L-cystine after a brief period of labeling, the M_r 9000 peak decreases and a corresponding amount of label appears in EGF. The M_r 9000 species was isolated from boiled homogenates in which it accounts for ≈1% of the total EGF content. It contains five of the six chymotryptic peptides of EGF and a sixth peptide which is a modified form of the COOH-terminal chymotryptic peptide of EGF. Of the arginyl esteropeptidases, γ subunit of 7S nerve growth factor, β-endopeptidase, trypsin, and EGF-binding protein, only the latter converts the isolated M_r 9000 species to EGF. The extrapeptide material released in the conversion comes from the COOH terminus of the M_r 9000 species. These results suggest that the M_r 9000 species is a biosynthetic precursor of EGF and that the EGF-binding protein is the specific intracellular cleaving enzyme that converts the precursor to EGF. In the process, the stable high molecular weight complex of EGF is formed.

Epidermal growth factor (EGF), a peptide that stimulates the growth of various cells and tissues (1), is found in the submaxillary gland of the adult male mouse (2). It can be isolated from the gland as a high molecular weight complex (HMW-EGF) in which each of the two EGF peptide chains are associated with a specific arginine esteropeptidase, the EGF-binding protein (EGF-BP) (3). The amino acid sequence of the EGF chains has been determined; they have a M_r of 6045 and COOH-terminal arginine residues (4). The COOH-terminal arginine residues are required for formation of HMW-EGF (5) but not for biological activity (6). Furthermore, the enzymatic activity of EGF-BP is inhibited in HMW-EGF, suggesting that in the complex the active sites of EGF-BP interact with the COOH-terminal arginine residues of EGF (5). These findings support the original hypothesis that the EGF chains are initially synthesized as higher molecular weight pro-EGF chains and that the extrapeptide material at the COOH terminus of the EGF chain is removed by the specific trypsin-like EGF-BP (3).

In the analogous β nerve growth factor (βNGF) system it has now been shown that the βNGF peptide chains are derived from longer pro-βNGF chains and that the trypsin-like γ subunit, the arginine esteropeptidase found associated with βNGF in its high molecular weight complex, 7S NGF, is one of the enzymes that carries out the conversion (7). The observation that, in spite of its remarkable similarity to the γ subunit, EGF-BP will not substitute for the latter in the formation of the 7S NGF complex suggests that the two enzymes should be specific for the processing of the appropriate growth factor precursors (8). It has not yet been possible to demonstrate this specificity of processing in the βNGF system, mainly because pro-βNGF has not been isolated in its native form. This has now been achieved in the EGF system. The evidence for the existence of pro-EGF is described in this paper. In addition, because of the stability of pro-EGF to heat, native pro-EGF has been obtained and the specificity of its cleavage by EGF-BP has been demonstrated.

MATERIALS AND METHODS

Protein and Antiserum. EGF was purified according to the method of Savage and Cohen (6). EGF antiserum was prepared in New Zealand White rabbits by injection of purified EGF in complete Freund's adjuvant (9). Antisera were judged to be monospecific by Ouchterlony double-diffusion analysis and immunoelectrophoresis. One microgram of EGF was precipitated by 80 μl of antiserum. L-[^{35}S]Cystine (specific activity 300 Ci/mmol; Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. The γ subunit of 7S NGF was isolated by the method of Smith *et al.* (10), and β-endopeptidase (a gift from W. Wilson) was prepared by the procedure of Wilson and Shooter (11).

Treatment of Animals. Adult male Swiss Webster mice were castrated and, at least 21 days later, were injected at 48, 24, and 1 hr before sacrifice with 1 mg of testosterone propionate in 0.2 ml of sesame oil (12). This procedure first decreased the EGF content of the submaxillary gland to ≈10% of normal and then increased the rate of EGF biosynthesis significantly.

***In Vitro* Incorporation Experiments.** Submaxillary glands were cut into small pieces and incubated at 37°C with 95% O_2/5% CO_2 in Dulbecco's modified Eagle's medium except that L-cystine was replaced with L-[^{35}S]cystine. The amounts of tissue and isotope, the volumes of medium, and tissue of incubation are indicated for each experiment. After incubation the tissue pieces were rinsed and homogenized in 1 ml of buffer per 100 mg of tissue (wet weight) by using a glass homogenizer with a medium-fitting Teflon pestle. The homogenization buffer contained 0.05 M sodium acetate buffer, 0.5 M NaCl, 1% Triton X-100, 0.1% bovine serum albumin, 200 μg of sodium azide and 50 μg of basic pancreatic trypsin inhibitor (Sigma)

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Abbreviations: EGF, epidermal growth factor; HMW-EGF, high molecular weight epidermal growth factor; EGF-BP, epidermal growth factor binding protein; βNGF, β nerve growth factor; pro-EGF and pro-βNGF, precursors to EGF and βNGF, respectively; NaDodSO$_4$, sodium dodecyl sulfate.

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per ml, and 5 mM phenylmethylsulfonyl fluoride. The final pH was 4.15. The homogenates were placed in a boiling water bath for 90 sec and then centrifuged at 105,000 × g for 60 min at 4°C. The resulting supernatant was assayed for labeled proteins precipitable by anti-EGF antiserum.

Isolation of EGF and EGF-Like Proteins by Immunoprecipitation. The quantity of antiserum required to precipitate the maximal amount of EGF was determined by titrating aliquots of supernatant containing ≈1 ng of ^{125}I-labeled EGF (^{125}I-EGF) with anti-EGF antiserum in 25 mM phosphate buffer at neutral pH. To a fresh aliquot (0.5 ml) of homogenate, 0.5 ml of 0.1 M phosphate buffer at pH 7.2 and the appropriate amount of antiserum were added. The precipitation was allowed to proceed overnight at 4°C. The precipitate was collected and washed three times in 0.01 M phosphate buffer at pH 7.2. The yield was 90% based on the recovery of ^{125}I-EGF.

Electrophoresis on Acrylamide Gel. The washed immunoprecipitates were dissolved in 0.06 ml of the sodium dodecyl sulfate ($NaDodSO_4$) sample buffer described by Laemmli (13), modified by the addition of 8 M urea. Then, 2% (wt/vol) dithioerythritol was added and the mixture was placed in a boiling water bath for 5 min. Analysis was on the discontinuous $NaDodSO_4$/polyacrylamide gel system described by Laemmli (13). The resolving gel containing 16.5% acrylamide and 0.44% *N,N'*-methylene bisacrylamide. After electrophoresis, the gels were cut into 2-mm slices and each slice was incubated at 37°C in 8 ml of scintillation fluid as described by Berger and Shooter (14).

Trichloroacetic Acid Precipitation. Aliquots (20 μl) of submaxillary gland supernatants were spotted (in triplicate) on Whatman GF-C filter discs. The filters were soaked batchwise at 4°C in 5 ml of 10% trichloroacetic acid for 30 min, in 95% ethanol for 5 min, and finally in anhydrous ether for 5 min. They were dried at room temperature and assayed for radioactivity.

Isolation of M_r 9000 Species from Submaxillary Glands. The procedure developed for isolating EGF by Savage and Cohen (6) was followed. Submaxillary glands from 50 male mice were homogenized in 40 ml of ice-cold 0.05 M acetic acid in a Waring Blendor for 3 min. The resulting crude homogenate was either placed in a boiling-water bath for 90 sec or, following the original procedure, frozen in a CO_2/acetone bath. The eluate from the Bio-Gel P-10 column was monitored for EGF and EGF-like material by radioimmunoassay (15). As Savage and Cohen (6) noted, one major immunoreactive peak containing EGF was observed together with a minor peak that eluted at ≈1 column volume of buffer. The material of this minor peak was concentrated and further purified on a DEAE-cellulose column (1.5 × 20 cm) equilibrated with 0.02 M ammonium acetate buffer (pH 5.6) at 5°C. The flow rate was maintained at 12 ml/hr. Elution of adsorbed protein was achieved with an ammonium acetate gradient: 0.2 M ammonium acetate at pH 5.6 was allowed to flow into a 125-ml constant volume mixing chamber initially containing 0.02 M ammonium acetate at pH 5.6. The eluate was monitored by measuring the absorbance at 280 nm and by radioimmunoassay or immunoprecipitation. Excluding protein eluted at the void volume, the M_r 9000 species was the first immunoreactive peak eluted from the column.

Iodination of EGF and EGF-Like Material. In order to determine the M_r of small quantities of EGF and EGF-like material, these proteins were labeled with ^{125}I. From 1 to 5 μg of protein was dissolved in 50 μl of 0.1 M phosphate buffer at pH 7.4, and 5 μl of lactoperoxidase (0.03 mg/ml), 5 μl of H_2O_2 (30%, diluted 1:10,000), and carrier-free Na ^{125}I (0.2 mCi) were added. The mixture was allowed to stand for 30 min at room temperature; then another 5 μl of the diluted H_2O_2 was added and the mixture was allowed to stand for another 30 min. The iodinated protein was separated from remaining free iodine by gel filtration on a Bio-Gel P-2 column (0.5 × 20 cm) equilibrated with 0.01 M phosphate buffer, pH 7.2/0.1% serum albumin. The fractions containing the iodinated protein were combined and stored frozen.

Analysis of the Chymotryptic Peptides of EGF and M_r 9000 Species. EGF and the M_r 9000 species (10–15 μg each) were oxidized with performic acid and digested with α-chymotrypsin (Worthington) according to Savage *et al.* (4). The resulting peptides were lyophilized and then dissolved in 10 μl of glacial acetic acid/formic acid (88%)/water, 150:50:800 (vol/vol). This mixture was also used as electrophoresis buffer. The peptides were spotted in one corner of a cellulose thin-layer chromatography plate (10 × 10 cm, Merck) and subjected to electrophoresis in the first dimension at 1000 V for approximately 20 min; fuchsin red was used as tracking dye. The plate was dried and then chromatographed in the second direction with *n*-butanol/H_2O/pyridine/glacial acetic acid, 65:40:50:10 (vol/vol). After drying, the peptides were stained either with Fluram (Roche Diagnostics, 2 mg in 30 ml acetone) or with ninhydrin.

NH_2-Terminal Labeling of the M_r 9000 Species. Approximately 2 μg of the M_r 9000 species was coupled with *N*-succinimidyl-3-(4-hydroxy, 5-[^{125}I]iodophenyl) propionate ("Tagit", Calbiochem) according to Bolton and Hunter (16). The labeled protein was separated from unreacted label on a Bio-Gel P-2 column (0.5 × 10 cm) equilibrated with 0.05 M phosphate buffer at pH 7.2.

RESULTS

Incorporation of [^{35}S]Cystine into EGF and EGF-Like Proteins in the Submaxillary Gland. The characterization of the immunoprecipitates from submaxillary glands labeled for various times is shown in Fig. 1. At the shorter times of incubation, the major fraction of the label appeared in a species with an apparent M_r of 9000. After ≈1 hr, the radioactivity in this species reached a plateau (Fig. 2). In contrast, radioactivity in the EGF position increased steadily after an initial lag and by 4 hr it exceeded that in the M_r 9000 species.

In a pulse–chase experiment in which the glands were labeled for 30 min and then either homogenized directly or further incubated in a large excess of unlabeled cystine, radioactivity increased initially in both the EGF and M_r 9000 species for a further 30 min (Fig. 3). After this, the radioactivity in the M_r 9000 species decreased and an almost equivalent amount of radioactivity appeared in the EGF position. Because incorporation of label into trichloracetic acid-precipitable material was decreasing over this same time period, these results suggest, as do those in Figs. 1 and 2, that the M_r 9000 species is being converted to EGF. Similar results were obtained with homogenates that were not boiled prior to immunoprecipitation, indicating that the M_r 9000 species is as heat stable as EGF. In unboiled homogenates, the amount of the M_r 9000 species decreased during storage at 4°C, presumably as a result of processing.

Specificity of the Processing of the M_r 9000 Species. Because of the heat stability of the M_r 9000 species, endogenous proteases in the gland homogenate could be inactivated by boiling and the processing of the M_r 9000 species then followed by addition of specific proteolytic enzymes to the cooled homogenate. The results of the incubation, of a boiled homogenate of glands labeled for 60 min, with various enzymes and then immunoprecipitated with anti-EGF antisera are shown in Fig.

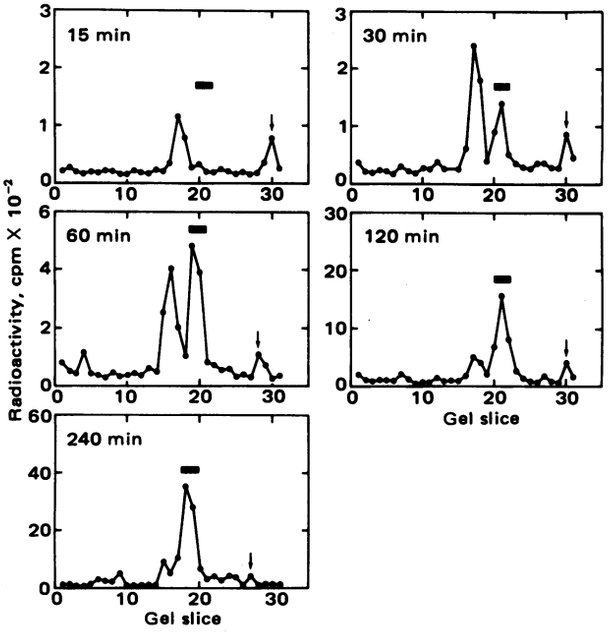

FIG. 1. Electrophoretic analyses, in NaDodSO$_4$/polyacrylamide gels, of the immunoprecipitates from continuously labeled glands. Submaxillary gland pieces (380 mg, wet weight) were incubated in 0.8 ml of cystine-free medium supplemented with 0.8 mCi of L-[^{35}S]-cystine. At the indicated times, ≈80 mg (wet weight) of tissue was processed. Arrows, position of the dye front; solid bars, EGF position, determined from the mobility of purified EGF analyzed on a separate gel and stained with Coomassie blue. Note the different scales on the ordinates.

4. The control homogenate with no added enzymes showed a major labeled species at M_r 9000 and a smaller peak in the EGF position. Addition of EGF-BP to the homogenate at a molar ratio of approximately 1.1:1 compared to the total EGF and M_r 9000 content of the homogenate resulted in the complete conversion of the M_r 9000 species to a species migrating in the position of EGF. In contrast γ subunit, β-endopeptidase, and trypsin at the same molar ratio had no effect on the M_r 9000 species. Even increasing the molar ratio of these three enzymes

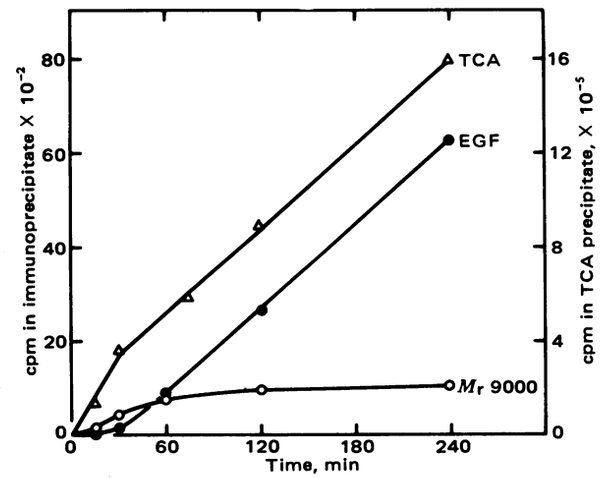

FIG. 2. Kinetics of labeling of the M_r 9000 species and EGF. Data were obtained from the experiment shown in Fig. 1 by calculating the total radioactivity in either the M_r 9000 species or EGF at each indicated time. Values represent radioactivity in 0.5 ml of supernatant. The upper curve (TCA) is the trichloroacetic acid-precipitable radioactivity in 20 μl of supernatant that was not heated before centrifugation.

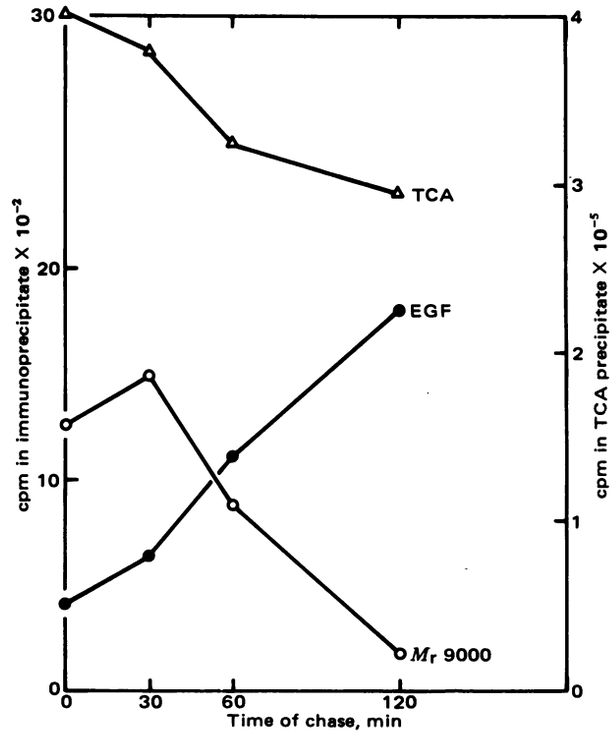

FIG. 3. Pulse–chase kinetics of the labeling of the M_r 9000 species and EGF. Submaxillary gland pieces (300 mg, wet weight) were incubated in 0.6 ml of cystine-free medium supplemented with 1.2 mCi of L-[^{35}S]cystine. After 30 min, tissue pieces were rinsed and either homogenized directly (no chase) or transferred into 2 ml of fresh medium containing 115 μg of unlabeled L-cystine per ml and no additional label. At the indicated times the gland pieces were processed, the immunoprecipitate was obtained from 0.5 ml of the supernatant, and the precipitate was analyzed by electrophoresis in NaDodSO$_4$/polyacrylamide gels. The total radioactivities in the 9000 M_r and the EGF peaks were calculated. The upper curve (TCA) represents trichloroacetic acid-precipitable radioactivity in 20 μl of supernatant obtained from gland homogenates that were not heated before centrifugation.

to 500:1 or increasing the time of incubation to 4 hr at 37°C did not result in cleavage of the M_r 9000 species. In contrast, reducing the molar ratio of EGF-BP to the total EGF and M_r 9000 content of the homogenate to 0.5:1 resulted in only about 50% conversion of the M_r 9000 species to EGF. This is the result that would be expected if the EGF-BP remains bound to the product of the cleavage event (EGF) and its activity is thereby inhibited.

Isolation and Characterization of the Native M_r 9000 Species. As Savage and Cohen (6) originally noted, a small peak of EGF-immunoreactive material is found in analyses of gland homogenate on a Bio-Gel P-10 column, eluting at approximately 1 column volume. Fractionation of this material on DEAE-cellulose gave three immunoreactive peaks containing material with M_rs of 9000, 6000, and 5600 as well as two other variable-sized immunoreactive peaks containing material of M_r 4700 and 3900. The isolated M_r 9000 species comigrated with the *in vitro* labeled M_r 9000 species in acrylamide gels containing NaDodSO$_4$. If it is assumed that the extinction coefficient for the M_r 9000 species is the same as for EGF (17), the M_r 9000 species accounts for about 0.3% of the total EGF isolated from untreated homogenates and for about 1% when the homogenate is boiled prior to its isolation. As noted earlier, this indicates that processing of the M_r 9000 species occurs continuously in homogenates.

The chymotryptic peptide map of the M_r 9000 species is

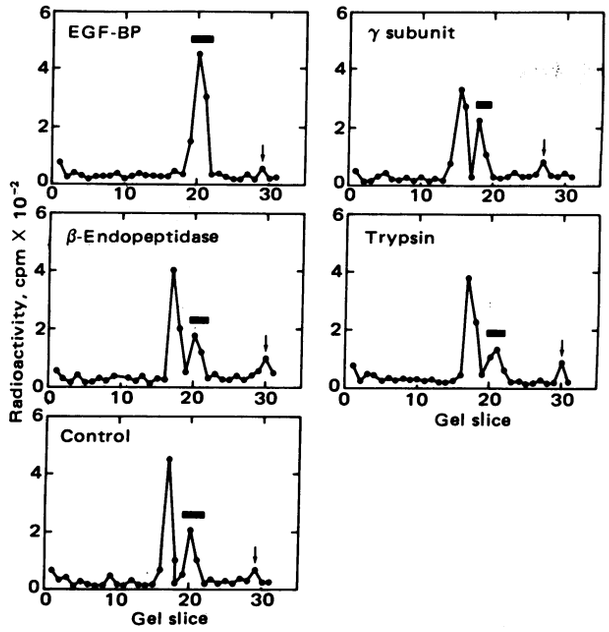

FIG. 4. Specificity of the cleavage of the M_r 9000 species. Submaxillary gland pieces (300 mg, wet weight) were incubated in 0.6 ml of cystine-free medium supplemented with 0.5 mCi of L-[^{35}S]cystine. After 1 hr the tissue pieces were rinsed and homogenized as described in *Materials and Methods* except that the buffer contained no protease inhibitors. The homogenate was placed in a boiling water bath for 90 sec, cooled, and centrifuged. The supernatant was divided into five 0.5-ml aliquots, each containing ≈12 μg of total EGF and M_r 9000 species. One sample served as a control and was added to 1.5 ml of 0.1 M phosphate buffer at pH 6.8. To the four remaining samples was added 65 μg of EGF-BP, γ subunit, β-endopeptidase, or trypsin, each in 1.5 ml of 0.1 M phosphate buffer at pH 6.8; these mixtures were incubated at 37°C for 2 hr. After immunoprecipitation, the precipitates were analyzed by electrophoresis in NaDodSO$_4$ gels. Solid bar, EGF position; arrow, position of the dye front.

compared with that of EGF in Fig. 5. Five of the six major peptides of the M_r 9000 species overlap major peptides from EGF. Six chymotryptic peptides were observed by Savage *et al.* (4), the most basic being derived from the COOH terminus of EGF. It is only this peptide that is modified in the M_r 9000 species, suggesting that the latter is identical to EGF except for modification at the COOH terminus.

The specificity of the processing of the isolated native M_r 9000 species was examined by using the enzymes described in Fig. 4. To follow the conversion of the M_r 9000 species ≈1 μg of ^{125}I-labeled M_r 9000 species was added before digestion with the enzymes, and the products were analyzed directly by

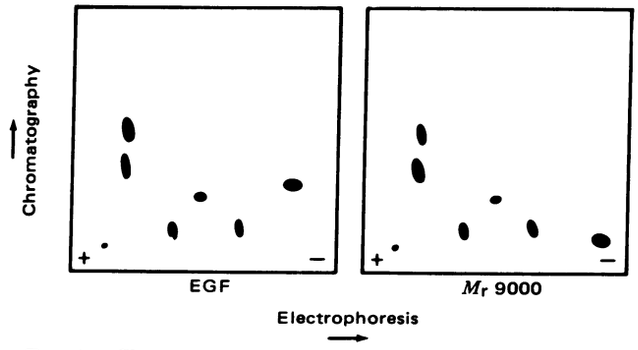

FIG. 5. Chymotryptic peptide maps of EGF and the M_r 9000 species. The sample was initially placed in the lower left corner of the plate.

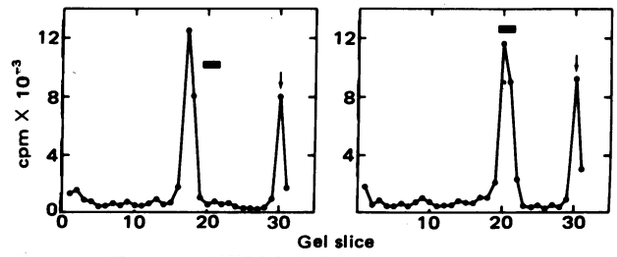

FIG. 6. Cleavage of ^{125}I-labeled M_r 9000 species with EGF-BP. The ^{125}I-labeled M_r 9000 species (0.5 μg) was incubated with 2.5 μg of EGF-BP in 20 μl of 0.1 M phosphate buffer (pH 6.8) for 2 hr at 37°C, added to 60 μl of the Laemmli sample buffer, and analyzed by electrophoresis in NaDodSO$_4$/polyacrylamide gels. (*Left*) Control sample incubated in the absence of enzyme; (*Right*) enzyme-treated sample. Solid bars, EGF position; arrows, dye front.

electrophoresis in NaDodSO$_4$/polyacrylamide gels. Only EGF-BP cleaved the M_r 9000 species to EFG and, again, large excesses of the three other enzymes failed to modify the M_r 9000 species. Moreover, when the products of the cleavage reaction were analyzed by gel filtration, ≈30% of the EGF was recovered in the form of HMW-EGF. Because analysis of authentic HMW-EGF at the same concentration and under the same conditions also resulted in recovery of 30% of the EGF as HMW-EGF (and the remaining 70% as free EGF), it is clear that the major product of the processing of the M_r 9000 species is HMW-EGF but that approximately two-thirds of it dissociates during the gel filtration.

In order to determine the location of the extrapeptide material in the M_r 9000 species the latter was treated with ^{125}I-labeled Bolton–Hunter reagent. Because EGF contains no lysine residues (4), only its NH_2-terminal amino group will be labeled with ^{125}I. If the extrapeptide material contains lysine, these residues will also be labeled. It follows that, if the extrapeptide material is released from the COOH terminus of EGF by the action of EGF-BP, the remaining EGF will still carry the ^{125}I-label. If extrapeptide material is released from the NH_2 terminus the remaining EGF will be unlabeled. Analysis of the released EGF (Fig. 6) showed that the label in the M_r 9000 species was quantitatively retained in EGF. The extrapeptide material is therefore released from the COOH terminus of the M_r 9000 species and contains no lysine residues.

DISCUSSION

The kinetics of the labeling of the M_r 9000 species in continuous labeling and pulse–chase experiments, its chymotryptic map, and its conversion to EGF by the EGF-BP all lead to the conclusion that the M_r 9000 species is a biosynthetic precursor of EGF—namely, pro-EGF. The extrapeptide material is released from the COOH terminus of pro-EGF and has a molecular weight of ≈3000. Failure to detect the extrapeptide material after cleavage of pro-EGF could result either from lack of resolution of the electrophoretic system in this size range or, more likely, from the absence of cystine residues in the extrapeptide material itself.

The specificity of the cleavage of pro-EGF by EGF-BP alone fits in well with the predictions of the model for the formation of the high molecular weight complex of EGF during the biosynthesis of EGF (Fig. 7). According to this model, the final stage in the biosynthesis of EGF is the cleavage of pro-EGF by the specific arginine esteropeptidase EGF-BP, with release of extrapeptide material from the COOH terminus of the precursor, exposing the COOH-terminal arginine residue of EGF. The selectivity of this processing event is now emphasized by the finding that the arginine esteropeptidase γ subunit from

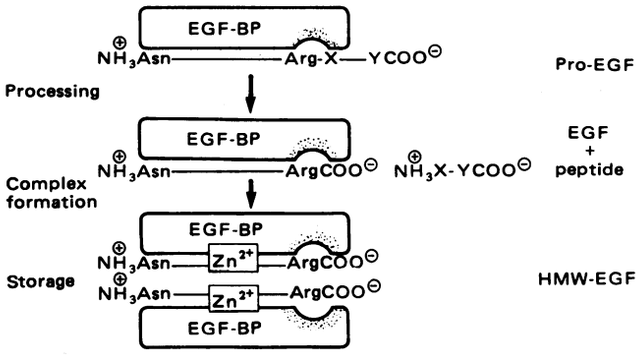

FIG. 7. Biosynthesis of EGF and the production of its high molecular weight complex. Recent observations (unpublished data) indicate that the complex contains zinc ions.

the 7S NGF complex does not convert pro-EGF to EGF. It is further emphasized by the failure of trypsin and β-endopeptidase to carry out this process although both are arginine esteropeptidases. The β-endopeptidase belongs to the same class of submaxillary gland arginine esteropeptidases as does γ subunit and EGF-BP, and one of its known functions is the release of the NH_2-terminal octapeptide sequence from native βNGF chains through hydrolysis of a histidine–methionine peptide bond (11). Thus, the selective cleavage of pro-EGF by EGF-BP offers strong support for the model.

Of equal importance are the observations that (*i*) the processing reaction requires stoichiometric rather than catalytic amounts of EGF-BP, (*ii*) when the ratio of enzyme to pro-EGF is less than 1:1, the conversion of pro-EGF is incomplete, and (*iii*) the processing of pro-EGF results in the formation of the HMW-EGF complex. All three findings fit with the predictions of the model (Fig. 7). The formation of a stable 1:1 complex between EGF-BP and its product, the EGF chain, results from the homology between the latter and the pancreatic trypsin inhibitor (18) and, as with the 7S NGF complex (19), provides a stable storage complex for the processed growth factor chain. It should be noted that the recovery of the HMW-EGF complex during the processing of pro-EGF was not quantitative because the conditions used for the cleavage reaction and the subsequent analysis of the products are not those that give maximal stability of the complex. The biosynthesis of EGF in the submaxillary gland therefore involves the cleavage of a biosynthetic precursor, pro-EGF, by a highly specific protease, EGF-BP, and the formation of a corresponding high molecular weight complex, HMW-EGF. It is known that the high molecular weight 7S NGF complex is produced in a similar manner (7) and all that remains is to determine if the specificity of the cleavage of isolated native pro-βNGF is as absolute as is that of pro-EGF.

The present data do not speak directly to the question of whether earlier precursor forms of EGF exist. However, because EGF is a secretory protein, it would not be unreasonable to assume that there is a pre-pro form, analogous to the various pre-pro secretory proteins that have now been characterized (20–22). The kinetics of the labeling of pro-EGF (Fig. 2) suggest that, like EGF, label may appear in this form after a short lag period. This may reflect the initial synthesis of an earlier precursor which is not solubilized under the conditions used or is not precipitated by the antiserum or is processed too rapidly to be detected. In addition, the acrylamide gel system may not be able to resolve the pre-pro- and pro-forms of EGF.

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