Intracellular Processing of Epidermal Growth Factor

II. INTRACELLULAR CLEAVAGE OF THE COOH-TERMINAL REGION OF 125I-EPIDERMAL GROWTH FACTOR

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Epidermal growth factor (EGF) undergoes a specific series of alterations during the course of its binding and internalization into cultured fibroblasts. The modified EGF species can be distinguished from each other and from native EGF by their isoelectric points. We employed peptide mapping techniques to determine the nature of these alterations. A [125I]-EGF with a PI of 4.55 was converted to a PI 4.2 species by removal of 1 or 2 amino acid moieties from the COOH-terminal end of the protein. A PI 4.35 species was generated by a trypsin-like cut between amino acid residues 48 and 49, for a total of 5 amino acid moieties removed from the native EGF. The PI 4.0 species was formed by removal of at least the COOH-terminal arginine from the PI 4.35 species. Thus, upon binding and internalization, EGF was sequentially cleaved in the COOH-terminal region. Removal of the COOH-terminal polypeptide has been shown to dramatically reduce the affinity of EGF for its receptor, raising the possibility that intracellular dissociation of EGF from its receptor may be a direct result of the intracellular processing of EGF.

Epidermal growth factor elicits its biological activity through a mechanism involving the binding of EGF to specific receptors on the surface of target cells. The receptor-EGF complexes form clusters on the plasma membrane and are endocytosed within clathrin-coated vesicles (1). During these early steps most bound [125I]-labeled EGF is converted from PI 4.55 to a PI 4.2 form (2, 3). The vesicles containing receptor-EGF complexes cofractionate on Percoll density gradients with markers for coated vesicles, Golgi, and endoplasmic reticulum. As shown in the accompanying paper (3), [125I]-EGF is converted in these vesicles to a PI 4.35 species. Eventually, the [125I]-EGF is modified to PI 4.0 and is found in dense, lysosomal-like organelles. The relationship of each of the preceding steps to the triggering of the biological response of cells to EGF remains unknown.

The modified [125I]-EGF species can be distinguished from the original [125I]-EGF by their isoelectric points and their affinities for EGF receptors but not by size as measured by chromatography on Sephadex G-75 columns (2). Thus, the altered EGF species may result from small additions or deletions such as by phosphorylation or limited proteolysis. The purpose of this investigation is to determine the precise nature of the EGF modification reactions.

We employed protein mapping techniques with proteolytic enzymes of known specificity in order to localize regions on the EGF molecule which are modified by intracellular processes. Since such studies clearly require a very pure ligand, we used α-EGF that had been purified to near homogeneity with reverse-phase high performance liquid chromatography as the final purification step (4). After an iodination reaction, the labeled EGF was purified by isoelectric focusing in agarose gels (5). Only the [125I]-EGF species with a PI of 4.55, the same as unreacted α-EGF, was used in these studies. Interpretation of our data has been aided by the amino acid sequence of mouse EGF originally published by Savage et al. (6) (Table I) and recently confirmed at the genetic level by Gray et al. (7).

We found that upon binding and internalization into Rat-1 fibroblasts, [125I]-EGF underwent a series of proteolytic cleavages near the COOH-terminal end of the protein.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat-1 cells (8) were propagated at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 2% newborn calf serum and equilibrated with 5% CO₂/95% air.

EGF Purification and Iodination—EGF was isolated from mouse submaxillary glands (9) and purified to near homogeneity by reverse-phase high performance liquid chromatography (4) as modified (3). The most abundant EGF species, α-EGF, was labeled with [125I] using chloramine-T (10). The iodinated form of EGF with an isoelectric point of 4.55, corresponding to the PI of unlabeled α-EGF, was purified by preparative isoelectric focusing on agarose gels as previously described (5). The specific activity of the [125I]-EGF was approximately 500 mCi/μg.

Isoelectric Focusing—Isoelectric focusing was performed with horizontal 1% agarose gels as previously described (2, 5). The pH range of the gels was approximately from 3.0 to 6.0. The isoelectric points of the various [125I]-EGF species were determined from standard curves generated with the aid of pI marker proteins (Federal Marine Colloids, Inc.) detected by Coomassie blue R250 staining. The location of the [125I]-protein was determined by autoradiography of analytical gels after drying and of preparative gels without drying (2).

Purification of Internalized [125I]-EGF Species—Ten 10-cm culture dishes of confluent Rat-1 cells (1 × 10⁶ cells/dish) were rinsed with Dulbecco's modified Eagle's medium. To each dish, 4 ml of binding media containing 3 × 10⁵ cpm/ml of [125I]-EGF and 10 mM methionine (to permit accumulation of internalized [125I]-EGF products) were added, and the cultures were incubated at 37 °C for 3 h. The dishes were rinsed 6 times in cold Hank's balanced salt solution plus 1 mg/ml of bovine serum albumin and the bound EGF was extracted from the cells with 5 ml/dish of cold 0.05 M HCl for 18 h. The dishes were rinsed with 5 ml of cold 0.05 M HCl, and the acid-soluble products were combined, extensively dialyzed against cold water, and lyophylized. The various internalized [125I]-EGF species were separated by preparative isoelectric focusing as described for the purification of [125I]-EGF (5).

Carboxypeptidase B Digestion—Reaction mixtures (100 μl) contained the indicated [125I]-EGF species, 5 μg of bovine serum albumin,
The PI 4.35 species was bound to a Beckman ultrapore RPSC purified by preparative isoelectric focusing for use as standards in peak having a PI of 4.3 and COOH-terminal amino acids consistent with peak eluting from the reverse-phase column. The protein in this third preparations, respectively.

The remaining 48 amino acids of EGF, EGF14; analysis of the amino acids released by carboxypeptidase A (Sigma) and carboxypeptidase B (Sigma, type 1, diisopropyl fluorophosphate-treated). The reactions were run at 37 °C for either 5 min or overnight and stopped by the addition of 50 μl of 0.2 M acetic acid. The samples were lyophilized prior to isoelectric focusing.

To determine the identity of the major tryptic fragments of 125I-EGF, we first digested EGF in the absence of bovine serum albumin and separated the products by reverse-phase high performance liquid chromatography as described for the preparation of α-EGF. Under the milder digestion conditions, 2 new peaks were detected by absorption of 280 nm light. One of the peaks was shown to be the COOH-terminal pentapeptide of EGF by digestion with aminopeptidase and analysis of the released amino acids after precolumn derivatization with o-phthalaldehyde (11). The protein comprising the second peak was the remaining 48 amino acids of EGF, EGF14; analysis of the amino acids released by carboxypeptidase A (Sigma) and carboxypeptidase B was consistent only with cleavage adjacent to the arginine moiety at position 48. Similarly, Savage et al. (6) reported that mild trypsin digestion of undenatured EGF selectively yielded EGF,14. Isoelectric focusing analysis showed that EGF14 was a pl of 4.35. Longer times of trypsin digestion of EGF resulted in a third protein peak eluting from the reverse-phase column. The protein in this third peak had a pl of 4.3 and COOH-terminal amino acids consistent with the protein being EGF,14. Both 125I-EGF14 and 125I-EGF4-14 were purified by preparative isoelectric focusing for use as standards in this study.

The identities of the minor trypsin fragments with pl values below 4 were not determined. These fragments may have resulted from slight oxidation or trace amounts of another proteolytic activity.

Fractionation of pl 4.35 Species by Reverse Phase Chromatography—The pl 4.35 species was bound to a Beckman ultrapore RPSC (C-3) column (4.6 × 75 mm) equilibrated in acetoni/trifluoroacetic acid, 15/85. The bound protein was eluted by raising the acetoni/trifluoroacetic acid proportion by 1%/min for 30 min. The flow rate was 1 ml/min, and 1-min fractions were collected. The 125I content of each fraction was measured in a γ counter. The samples in fractions 5 to 9 and 10 to 14 were pooled to form the pl 4.35a and pl 4.35b preparations, respectively.

RESULTS

A typical isoelectric point distribution of intracellular 125I-EGF species 40 min after internalization is shown, along with a sample of the original 125I-EGF, in the isoelectric focusing gel pictured in Fig. 1A. Clearly, the 125I-EGF has been converted to a few discrete species with very acidic isoelectric points. To investigate the nature of the changes we first separated the major intracellular 125I-EGF species by preparative isoelectric focusing. The most abundant iodinated species, with pl values of 4.55, 4.35, 4.2, and 4.0, were obtained with very little cross-contamination (Fig. 1B).

The cell-associated 125I-species which focused at pH 4.55 had the same pl as the starting 125I-EGF. The pl 4.55 species was the predominant form found on the cell surface and nearly disappeared from the cell within 15 min after internalization (3). Digestion of 125I-EGF or the pH 4.55 species with carboxypeptidase B resulted in a shift of the pl of the 125I-protein to 4.2 (Fig. 2). Apparently, both proteins had COOH-terminal arginine moieties; EGF contains no lysine moieties. Likewise, the 125I-products resulting from either mild or extensive trypsin digestion of the two proteins were indistinguishable by isoelectric focusing. Thus, the pl 4.55 species appears to be unmodified 125I-EGF.

The pl 4.2 species appeared on the cell surface within 10 min after binding of 125I-EGF (3). The autoradiographic picture in Fig. 3 shows that the pl 4.2 species had the same isoelectric point as 125I-EGF after treatment with carboxypeptidase B to remove the COOH-terminal arginine moiety. Furthermore, the pl 4.2 species was insensitive to treatment with carboxypeptidase B. Thus, the pl 4.2 species appears to have been formed by cleavage of the COOH-terminal arginine moiety (see Table I for amino acid sequence). The penultimate amino acid, leucine, may also have been removed. Since the leucine side chain is uncharged, its presence or absence cannot be detected with isoelectric focusing gels. The major product from mild digestion of 125I-EGF with Staphylococcus aureus V8 protease, which should release the COOH-terminal dipeptide, is indistinguishable on the isoelectric focusing gels from the carboxypeptidase B product (data not shown). In contrast, the glutamate moiety at position 51 must not have been

| Table I |

| Amino acid sequence of mouse EGF |

| NH₂-Asn-Ser-Tyr-Pro-Gly-Cys-Pro-Ser-Ser-Tyr-Asp-Gly-Tyr-Cys-Leu- |
| 5 | 10 | 15 |
| NH₂-Gly-Gly-Gly-Val-Cys-Met-His-Ile-Glu-Ser-Leu-Asp-Ser-Tyr-Thr- |
| 20 | 25 | 30 |
| Cys-Asn-Cys-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg-Gly-Gln-Thr-Arg- |
| 35 | 40 | 45 |
| Asp-Leu-Arg-Trp-Trp-Glu-Leu-Arg-COOH |
| 50 | 54 |

Data from Refs. 6 and 7. The lines indicate the portion of EGF missing in each of the indicated pl species.
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**CARBOXYPEPTIDASE B**

| UNDIGESTED | 5 MIN | 18h |
|------------|-------|-----|
| a          | b     | a   |
| a          | b     | a   |

**TRYSIN**

| UNDIGESTED | 5 MIN | 18h |
|------------|-------|-----|
| a          | b     | a   |
| a          | b     | a   |

FIG. 2. Isoelectric focusing gel analysis of proteolytic digests of the pI 4.55 species. The pI 4.55 species and \(^{125}\text{I}-\text{EGF}\) were incubated with the indicated proteases, and the products were resolved by isoelectric focusing, as described under "Experimental Procedures." Lane a, \(^{125}\text{I}-\text{EGF} ;\) lane b, pI 4.55 species.

removed, since this would have appreciably raised the pI of the protein. A comparison of the tryptic peptides generated from the pI 4.2 species and \(^{125}\text{I}-\text{EGF}\) suggests that only the COOH-terminal portion of EGF was altered in the formation of the pI 4.2 species; the labeled tryptic peptides were identical.

The second modified \(^{125}\text{I}-\text{EGF}\) species to appear in cells had a pI of 4.35 and was found in vesicles with the same buoyant density as clathrin-coated vesicles, Golgi, and endoplasmic reticulum (3). Upon digestion of the pI 4.35 species with carboxypeptidase B, under conditions where \(^{125}\text{I}-\text{EGF}\) reacted quantitatively, most the labeled protein underwent a pI shift indicative of the loss of the COOH-terminal arginine moiety, but a portion of the labeled protein was resistant to the treatment (Fig. 4). The resistant portion of the pI 4.35 species did not change pI even after prolonged incubation in the presence of carboxypeptidase B. Therefore, the pI 4.35 species as resolved by isoelectric focusing must be heterogeneous.

The two forms of the pI 4.35 species were partially separated by reverse-phase chromatography on a Beckman RPS (C-3) column and termed pI 4.35a and pI 4.35b species, respectively. The equality of the isoelectric points of the two species is evident in Fig. 5. When the two pI 4.35 preparations were incubated with carboxypeptidase B, about half of the labeled protein in the pI 4.35a sample shifted pI while essentially none of the pI 4.35b species was altered. Thus, the pI 4.35a species had a COOH-terminal arginine moiety and was contaminated with the pI 4.35b species which did not terminate in arginine. \(^{125}\text{I}-\text{EGF}\) characteristically forms broad peaks upon reverse-phase chromatography so we did not try at this time to improve resolution of the two species.

The pI 4.35 species had the same isoelectric point as a trypsin fragment consisting of amino acids 1 to 48 of \(^{125}\text{I}-\text{EGF}\), termed \(^{125}\text{I}-\text{EGF}_{1-48}\) (Fig. 5). The pI 4.35a species underwent the same pI shift as \(^{125}\text{I}-\text{EGF}_{1-48}\) upon digestion with carboxypeptidase B. Thus, it appears that the pI 4.35a and \(^{125}\text{I}-\text{EGF}_{1-48}\) are identical. The pI 4.35b species may have resulted from the incomplete removal of the tryptophan moieties at positions 49 and 50. In support of this interpretation is the protein profile from the RPS column indicating that the 4.35b species was more hydrophobic than the pI 4.35a species. Furthermore \(^{125}\text{I}-\text{EGF}_{1-48}\) was the principal product formed from EGF by very mild trypsinization conditions (Figs. 2 and 3) and both pI 4.35 species were resistant to mild trypsin digestion (Fig. 5). More vigorous trypsinization conditions generated the same labeled peptides from the \(^{125}\text{I}-\text{EGF}_{1-48}\) and the pI 4.35 species.

A pulse-labeling experiment, similar to those described in the accompanying paper (3), indicated that the percentage of intracellular \(^{125}\text{I}\) associated with the pI 4.35a species reached a peak between 20 and 40 min after a 5-min labeling period with \(^{125}\text{I}-\text{EGF}\). In contrast, the percentage of \(^{125}\text{I}\) associated with the pI 4.35b species remained constant for 4 h. Therefore, the pI 4.35a species and not the pI 4.35b species appears to be an intermediate in the processing scheme.

The pI 4.0 species is the last to appear and the longest lived of the major intracellular \(^{125}\text{I}-\text{EGF}\) species. It is found in dense, lysosomal-like organelles (3). The pI 4.0 species had

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the same isoelectric point as aliquots of the PI 4.35a species that had been digested with carboxypeptidase B to remove the COOH-terminal arginine moiety (Fig. 6). Thus, the PI 4.0 species could have been formed in the cell by removal of the COOH-terminal arginine or arginine-leucine moieties of the PI 4.35a species. Accordingly, the data in Fig. 6 indicate that the PI 4.0 species did not have COOH-terminal arginine. If the PI 4.0 species consisted of EGF amino acids 1 to 46 or 47, extensive trypsin digestion might be expected to generate a PI 4.3 species that we did not observe. However, the rate of hydrolysis by trypsin between positions 45 and 46 of the PI 4.0 species may have been greatly reduced by the acidic aspartate moiety adjacent to the arginine moiety and by the proximity of the arginine moiety to the COOH terminus.

DISCUSSION

We have previously reported that 125I-EGF is chemically altered as it traverses through recipient cells and that these alterations can be detected by isoelectric focusing (2, 3). It was not feasible to purify adequate quantities of the individual internalized 125I-EGF species for direct amino acid sequencing. However, we were able to localize the regions of change with peptide mapping techniques. We found that the 125I-EGF was sequentially cleaved near its COOH-terminal end to generate the altered species. The points of cleavage are indicated with the amino acid sequence of mouse EGF in Table I. Within a
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few minutes after 125I-EGF binds to cell surface receptors, the pl 4.2 species was formed by removal of 1 or 2 amino acid residues. This first cleavage occurred at least in part at the cell surface but also may have occurred after internalization (3). As the pl 4.2 species was transported through the cells in vesicles, an 125I-EGF form with a pl of 4.35 was produced by cleavage adjacent to the arginine moiety at position 48. The final major intracellular species, which is located in lysosomal-like organelles (3), had a pl of 4.0 and had lost at least the arginine moiety at position 48. The pl values described for each species agree well with the pl values calculated based on the presumed amino acid composition and textbook values for the pK values of the charged groups. We cannot eliminate the possibility of additional modifications within 125I-EGF that do not appreciably change the isoelectric point.

Both EGF and the EGF receptor appear to be cleaved upon binding of EGF at the cell surface (13, 14); EGF is cleaved more after internalization and the receptor also may be further cleaved. Any of the resulting fragments could conceivably serve as a second messenger to convey a signal within the cell. Another possibility, not necessarily exclusive from the latter, is that some of the biological responses could result from the activation of a tyrosine protein kinase activity upon binding of EGF to its receptor protein (12). Because removal of the COOH-terminal pentapeptide of EGF reduces the affinity of the EGF for its receptor (3, 15), this cleavage may cause EGF to separate from the receptor protein and thereby inactivate the receptor protein kinase activity. The freed EGF receptor may then migrate to another site within the cell or be degraded.

During the course of EGF binding and internalization, a diversity of signals may be generated to induce early and late cellular responses, including the modulation of de novo EGF receptor synthesis. Coordination of these responses may result from intracellular signals produced as a result of proteolytic cleavage of EGF and its receptor. Further experimentation is necessary to resolve this issue.

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