Intracellular Processing of Epidermal Growth Factor and Its Effect on Ligand-Receptor Interactions*

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H. Steven Wiley‡‡, William VanNostrand‡§, Dana Noel McKinley‡‡, and Dennis D. Cunningham‡‡

From the ‡Department of Pathology, the University of Utah Medical Center, Salt Lake City, Utah 84132 and the §Department of Microbiology and Molecular Genetics, College of Medicine, the University of California, Irvine, California 92717

When normal human fibroblasts are brought to a steady state with 125I-labeled epidermal growth factor (125I-EGF), greater than 90% of the radioactivity is intracellular. We investigated this material to determine whether the 125I-EGF is intact or degraded. Our results show that 125I-EGF is rapidly processed after internalization and can be resolved into four peaks by native gel electrophoresis. These different forms were isolated and tested for their ability to bind to cell-surface EGF receptors. The first processed form was fully capable of binding to EGF receptors, but the second processed form could not. The third form was a collection of small degradation products. We calculated that at steady state about 10% of the internalized 125I-EGF was in a form still able to bind to EGF receptors. We then investigated the ability of different reported inhibitors of EGF "degradation" to block the processing of EGF. Although inhibitors of cathepsin B (leupeptin, antipain, N-p-tosyl-L-lysine chloromethyl ketone, and chymostatin) were able to inhibit the release of monoiodotyrosine from treated cells in a concentration-dependent manner, they had little effect on the processing step that apparently inactivates 125I-EGF. In contrast, agents that raised intravesicular pH, such as methylamine and monensin, inhibited the initial steps in EGF processing as well as the later steps. Low temperatures inhibited the transfer of 125I-EGF to the lysosomes and inhibited the conversion of 125I-EGF to a nonbindable form, but had little effect on the initial processing. We conclude that the intracellular processing of EGF is a multistep process that is initiated prior to lysosomal fusion, involves cathepsin B activity, and requires an acidic pH. In addition, many of the protease inhibitors that have been utilized to investigate the role of EGF degradation in mitogenesis do not block the conversion of EGF to a form that is apparently unable to interact with its receptor.

The interaction of epidermal growth factor (EGF) with target cells is a complex process which involves binding, internalization, and degradation of the hormone (1, 2), as well as phosphorylation and processing of its cell-surface receptor (3–5). Although studies have shown that EGF binding to cell-surface receptors is necessary for the mitogenic response (6, 7), little is known about the role of these other events in the biological response. For example, it is not clear whether internalized EGF participates in the generation of the mitogenic signal. Some investigators have approached this issue by utilizing various inhibitors of EGF degradation and then determining their effect on EGF-stimulated mitogenesis (8–10). These studies have led to conflicting conclusions regarding the role of intracellular EGF since some inhibitors appear to either facilitate (8) or inhibit (9) some actions of EGF while other inhibitors have no apparent effect (10). In all of these studies, the ability of the inhibitors to block EGF degradation was assessed mainly by their ability to block the release of mono- and di[125I]iodotyrosine from treated cells. However, information is lacking about intermediate forms of EGF that might be formed during its intracellular processing, the potential activity of these intermediates, and the effect of inhibitors on their formations. These issues are important in view of our previous findings that during the induction of the mitogenic response, up to 92% of EGF associated with responsive human fibroblasts is in an intracellular compartment (11, 12).

These considerations prompted us to examine the nature of intracellular 125I-EGF and to evaluate its potential biological activity. For these analyses, we employed native gel electrophoresis which enabled us to identify several processed forms of EGF. We also investigated the ability of previously reported "inhibitors" of EGF degradation to block the different steps in EGF processing. The potential activity of the different forms was evaluated by analyzing their ability to interact with cell-surface EGF receptors. We found that the processing of EGF is initiated prior to delivery of the ligand to the lysosomes and that some agents that block the complete breakdown of EGF to amino acids do prevent intermediate steps that apparently destroy its ability to bind to the EGF receptor.

MATERIALS AND METHODS

RESULTS

Shown in Fig. 8 is a summary of our experiments on the different inhibitors of EGF "degradation." The indicated in...
In the present study, we have documented the intracellular processing of $^{125}$I-EGF and have also measured the effect of this processing on the ability of EGF to bind to its receptor. Subsequent to the completion of this study, a report appeared documenting the intracellular processing of EGF in RAT-1 cells (38, 39). That report showed that the initial processing of $^{125}$I-EGF involves the removal of several amino acid residues from the carboxyl terminus of the molecule, but it did not address the effect that the processing had on ligand-receptor interactions (39). The results of our study in general support the findings of Planck et al. (39) in that the migration position of the processed $^{125}$I-EGF on native gels is very similar to that reported for EGF in which up to 5 residues have been removed from the carboxyl terminus. In addition, we have analyzed the molecular weights of the different processed forms of $^{125}$I-EGF using high performance liquid chromatography under reducing conditions and in the presence of 6 M guanidine hydrochloride (results not shown). Those results indicate that peak II and peak III are virtually indistinguishable from native EGF with respect to molecular weight while peak IV EGF is a collection of small peptides. This is consistent with the initial processing involving a very small alteration in the number of amino acid residues of the protein as would occur if only a few amino acids were removed from the carboxyl terminus. However, the exact nature of the processing which occurs after EGF internalization in human fibroblasts can be established only after sufficient amounts of material for direct protein analysis are isolated. Such studies are currently in progress.

The initial step in the processing of $^{125}$I-EGF that we observed apparently occurs after binding and internalization since the addition of $^{125}$I-EGF directly to solubilized cells did not result in any alteration in the molecule (Fig. 1). In addition, when we bound $^{125}$I-EGF to the surface of cells at 0°C and then homogenized and fractionated the cells on Percoll gradients, all of the label sedimenting with the plasma membranes was intact $^{125}$I-EGF (data not shown). We found that initial processing of the $^{125}$I-EGF occurred within 5 min after entry into the cell (Fig. 1). This is prior to the time that internalized EGF has been observed to enter the lysosomal compartment (19, 40). Indeed, when we blocked the transfer of EGF to the lysosomes by lowering the incubation temperature, the initial processing of the EGF to peak II material was unaffected (Fig. 8). However, the conversion of peak I into peak II was pH-sensitive, as would be expected for a lysosomal process. It has been reported that there is a rapid acidification of endocytic vesicles (41). If there were pH-sensitive proteases or processing enzymes localized in endocytic vesicles, then the acidification of these vesicles might activate them. Alternatively, monensin and methylamine may act by inducing vesicular swelling (24, 25) which could then inhibit the transfer of $^{125}$I-EGF to vesicles where processing occurs.

In contrast to the initial processing of $^{125}$I-EGF, the conversion of peak II material to peak III and peak IV material had all the properties of a lysosomal process. There was a delay between the initial entry of EGF into cells and the initiation of degradation which is similar to the delay that has been observed between the endocytosis of EGF and the transfer to the lysosomes (19, 40). Inhibitors of the lysosomal protease cathepsin B (i.e. leupeptin) inhibited the later steps in EGF processing (Fig. 5). These later steps were also more sensitive to agents that raised intravesicular pH than the initial processing steps (Fig. 5). Finally, blocking the transfer of $^{125}$I-EGF to the lysosomes by lowering the incubation temperature of the cells also blocked the later steps in processing. Thus, it seems that the initial steps in EGF processing occur very shortly after internalization while the subsequent steps occur after the transfer of EGF to the lysosomes.

One of the aims of this study was to determine if internalized EGF was capable of occupying its receptor. We found that the processing of peak I to peak II EGF did not significantly alter its ability to bind to the EGF receptor (Fig. 3). In contrast, we found no measurable binding of peak III material to EGF receptors. The peak IV material could not be tested for its ability to bind to the cell surface since it was lost from the dialysis tubing. However, it is doubtful that it would bind in view of the inability of the larger peak III material to bind. From the present results, we can calculate that at steady state approximately 67% of the total radioactivity associated with human foreskin fibroblast cells is present in a form that can bind to the EGF receptor. Of this total, 13% is associated

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Fig. 8. Summary of the effects of inhibitors of EGF degradation on the intracellular processing pathway. Each inhibitor is shown next to the step in the processing pathway that is the most sensitive to it. When two steps are almost equally sensitive, then the inhibitors are shown next to both steps.

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*H. S. Wiley and D. J. Knauer, unpublished observations.*
with the surface of the cells, with the remainder in an internal compartment. We can thus conclude that a large majority of the EGF that is associated with responsive cells at steady state is in an intracellular compartment and is capable of binding to the EGF receptor.

It may be significant that the processing of EGF to a form incapable of interacting with its receptor occurs only after the transfer of the ligand to the lysosomes. It has been reported that the rate at which EGF is transferred to the lysosomes in human fibroblasts is significantly different from that observed for other internalized ligands (29). Thus, the termination of the ligand-receptor complex may be regulated by the rate of lysosomal transfer and not by the rate of its internalization. Another candidate for terminating the EGF-receptor complex is an acidification of the intracellular vesicle (41) which could cause dissociation of EGF from the receptor (15). Recently, we determined the minimum concentration of EGF inside endocytic vesicles by simultaneously measuring the internalization of [125I]-EGF and the internalization of [3H]-polypep

An unexpected finding of this study was that many agents that have been previously employed to inhibit the intracellular breakdown of EGF do not prevent intracellular processing of EGF in the hormonal response (10). Those studies found no significant effect of leupeptin on the cellular response to EGF. Is the intact intracellular EGF still associated with its receptor? Since EGF and its receptor are capable of signalization of '251-EGF and the internalization of '251-polyvinylpyrrolidone (42). These studies showed that the concentration of peak I1 to peak I11 forms (Fig. 5). Since we found that the acidification of the vesicle could then prevent reoccupancy of the receptor.

REFERENCES
1. Carpenter, G., Lembach, K. J., Morrison, M. M., and Cohen, S. (1975) J. Biol. Chem. 250, 4299-4305
2. Haigler, H. A., Ash, J. F., Cohen, S., and Singer, S. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3314-3318
3. Hunter, T., and Cooper, J. A. (1981) Cell 24, 741-752
4. Das, M., and Fox, C. F. (1975) Proc. Natl. Acad. Sci. U. S. A. 75, 2644-2648
5. Stoscheck, C. M., and Carpenter, G. (1984) J. Cell Biol. 98, 1048-1053
6. Aharown, A., Prus, R. M., and Herschman, H. R. (1978) J. Biol. Chem. 253, 3968-3977
7. Haigler, H. T., and Carpenter, G. (1980) Biochim. Biophys. Acta 96, 314-323
RT-PCR detected the expression of a number of genes encoding proteins relevant to cancer progression, indicating a potential role for these genes in the development of the disease.
Intracellular Processing of EGF

The above results indicate that not all of the internalized 125I-EGP is intact. This finding is consistent with the results of other investigators in whom inhibitors of EGF degradation have been used to probe the role of intracellular EGF degradation in the regulation of cellular responses. Since those studies used the release of 125I-labeled EGP as a criterion for EGF degradation, it is possible that the inhibitors utilized did not prevent processing of EGP into non-labile forms. To clarify these questions raised by previous studies on the role of intracellular EGF degradation, we examined a number of inhibitors of the degradation of 125I-EGP in our system.

We incubated confluent cultures of NIH 3T3 cells with 8.3 x 10^-10 M 125I-EGP and the indicated concentration of leupaptin, antipain, pepstatin, aprotinin, and capthatin as described in Experimental Procedures. The labeled EGP was then chased with medium containing the indicated inhibitors, and the amount of radioactivity associated with each peak was quantitated by scintillation counting. The results are expressed as the percentage of the total radioactivity recovered in the gel.

Since leupaptin does not prevent the intracellular processing of intact 125I-EGP, we were interested in the effect of other reported inhibitors of EGF degradation on this process. In Fig. 6, we present the results of such experiments in which cells were treated with either leupaptin or TLE or both, and the percentage of total radioactivity present in the intact peak was determined as described in Materials and Methods. The data presented in Table 1 show that the treatment of cells with leupaptin resulted in a decrease in the percent of intact EGP, whereas the treatment with TLE resulted in an increase in the percent of intact EGP. These results suggest that the processing of EGP is inhibited by leupaptin and TLE and that the percentage of intact EGP is increased by the treatment with TLE.

To further probe the role of an acidic intravesicular environment in the processing of EGP, we investigated the effect of the leupeptin on this process. It has been demonstrated that leupeptin will disrupt an acidic intravesicular pH by catalyzing the exchange of H+ for Na+ across membranes (20-23). Thus, if the processing of EGP depends upon an acidic intravesicular pH, we would expect to observe a decrease in the percent of intact EGP upon treatment with leupeptin.

The results of these experiments are presented in Table 1. As shown in Table 1, leupeptin resulted in a significant decrease in the percent of intact EGP, whereas TLE resulted in an increase in the percent of intact EGP. These results suggest that the processing of EGP is inhibited by leupeptin and TLE and that the percentage of intact EGP is increased by the treatment with TLE.

To further investigate the role of an acidic intravesicular environment in the processing of EGP, we examined the effect of leupeptin on the percent of intact EGP. As shown in Table 1, leupeptin resulted in a significant decrease in the percent of intact EGP, whereas TLE resulted in an increase in the percent of intact EGP. These results suggest that the processing of EGP is inhibited by leupeptin and TLE and that the percentage of intact EGP is increased by the treatment with TLE.

In conclusion, the results of these experiments suggest that the processing of EGP is inhibited by leupeptin and TLE and that the percentage of intact EGP is increased by the treatment with TLE. These results support the hypothesis that the processing of EGP is inhibited by leupeptin and TLE and that the percentage of intact EGP is increased by the treatment with TLE.
It was possible that monensin blocked the degradation of EGF not by raising intravesicular pH, but by raising the intravesicular Na content since the ionophore Catalyzed the exchange of Na across membranes. However, while monensin inhibited the degradation of EGF, the ionophore was ineffective in blocking the release of EGF degradation products from treated cells; their relative effectiveness was in the same order as their selectivity for Na (inhibitory > ionophore > monensin). To test for a possible role of Na in the effect of the inhibitors we treated cells with valinomycin. This ionophore will catalyze the exchange of Na across membranes if there is a passive Cl flow, since this has been shown to be the case with anionic vesicles (18). Valinomycin should cause a unidirectional exchange of Na, and the release of EGF degradation products from treated cells; their relative effectiveness was in the same order as their selectivity for Na (inhibitory > ionophore > monensin). Valinomycin had no effect on the ability of the cells to degrade internalized carboxylic ionophores inhibit the processing and degradation of the intravesicular level to rise significantly. Steps. This indicates that all of the steps in the intracellular processing vesicle itself prior to the delivery of the ligand to the lysosomes. The processing does not seem to occur at the cell surface since the initial processing step was blocked when internalization was inhibited by heparin, while cells incubated with labeled ligand for 5 min at different temperatures were incubated with labeled ligand for 5 min at different temperatures and then chased with unlabeled ligand for an additional hour. They were then homogenized and fractionated on Percoll gradients. Results from this experiment are shown in Table I, and indicate that blocking the transfer of EGF to the lysosomes was significantly inhibited by inhibitors. A temperature of 0°C was significantly higher than what has been reported to block ligand delivery to the lysosomes in other cell types. Therefore, it seemed possible that an acidic environment in the endosome was responsible for the initial processing of EGF if this is the case, then blocking the transfer of EGF to the lysosomes should have little effect on the initial processing step, but would strongly inhibit the latter steps.

It has been reported that lowering the incubation temperature of cells will block the transfer of internalized ligands to the lysosomes (29,30). To test this possibility, we treated cells with inhibitors and preincubated them with labeled ligand for 5 min with the inhibitors and then replaced with medium containing the inhibitor of the peak I to peak II conversion while lower temperatures are the most effective inhibitors of the peak III to peak IV conversion.

**Figure 6.** Low temperature blocks the transfer of $^{125}$I-EGF to the lysosomes. Confluent cultures of cells were preincubated at the indicated temperatures for 10 min and then incubated with EGF at 37°C for 5 min. The labeled EGF was removed and then replaced with the same concentration of unlabeled ligand for an additional hour at the indicated temperatures. The cells were then rinsed at 4°C and surface-associated ligand removed by a brief treatment with glycine-HCl buffer as described (41). Cells were then fractionated on 21 Percoll gradients as described in Experimental Procedures. The top and the gradients (lower density) are to the right of the panels. Shown are the data from one of the experiments is shown in Table II. At this point our experiments indicated that different inhibitors of EGF degradation inhibited different steps in the processing pathways. To directly compare their relative effectiveness on the different processing steps, we incubated cells with the different inhibitors, pulsed the cells with $^{125}$I-EGF, and then chased the cells with unlabeled ligand. We then compared the relative rates of the different forms of EGF. If an inhibitor blocked the transition from peak I to peak II, for example, then the relative ratio of peak II/peak I should be increased. A summary of our experiments is shown in Table II. We found that our inhibitors did not block the transfer of EGF from peak I to peak II conversion while lower temperatures are the most effective inhibitors of the peak III to peak IV conversion.

**TABLE III**

Effect of different inhibitors on the processing of $^{125}$I-EGF

| Treatment | Peak Ratio |
|-----------|------------|
| None      | 1/II       |
| Laeovipin (50 µg/ml) | 0.46 | 30.1 |
| Leucovorin (50 µg/ml) | 0.81 | 12.0 |
| Mesotriazin (10 mM) | 0.34 | 12.5 |
| TQCE (1.14 mEq/l) | 0.59 | 21.7 |
| TQDF (1.65 mEq/l) | 0.56 | 9.9 |

**Figure 7.** Low temperature blocks the transfer of $^{125}$I-EGF to the lysosomes. The internalized ligand would block the transfer of $^{125}$I-EGF to the lysosomes, allowing the processing of the peak III and peak IV forms of the molecule; however, the processing of the peak II form was unaffected, indicating that the initial processing of the ligand occurs prior to its delivery to the lysosomes.