Post-translational Processing of the Epidermal Growth Factor Receptor

GLYCOSYLATION-DEPENDENT ACQUISITION OF LIGAND-BINDING CAPACITY*

(Received for publication, September 4, 1984)

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The post-translational processing of the epidermal growth factor receptor in human A431 epidermoid carcinoma cells has been investigated. By employing the affinity matrix epidermal growth factor Affi-Gel in conjunction with immunoprecipitation, it has been demonstrated that core oligosaccharide addition is essential for the acquisition of epidermal growth factor-binding activity. Furthermore, the initial 160-kDa translation product was observed to undergo a processing step by which ligand-binding activity was acquired with a half-time of approximately 30 min while exhibiting no apparent change in mobility on sodium dodecyl sulfate-polyacrylamide gels. This was shown not to involve the conversion of high-mannose chains to complex chains which have been capped with fucose and sialic acid. Possible explanations for this activation in terms of translocation of intermediates and/or formation of disulfide bonds are discussed.

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Epidermal growth factor is a peptide hormone which is a potent mitogen for a wide variety of cultured cells. Its biological effects, which are mediated by binding to specific cell-surface receptors, include stimulation of ion transport, enhancement of endogenous protein phosphorylation, alterations in cellular morphology, and stimulation of DNA synthesis (1-3). Upon binding of EGF, the ligand-receptor complex is rapidly internalized (1, 3) and EGF is apparently degraded lysosomally, although the exact fate of the receptor is unknown. It remains to be determined whether internalization of the receptor is necessary to mediate the biological response through an intracellular mechanism (4). The EGF receptor also possesses a ligand-stimulated tyrosine-specific protein kinase activity which places it in a class of membrane glycoproteins including the receptors for insulin (5), insulin-like growth factor I (6), and platelet-derived growth factor (7).

Although the functional significance of this activity has yet to be elucidated, the ability to phosphorylate on tyrosyl residues is shared with at least 5 putative transforming proteins whose oncogenes are related to v-src (8). Furthermore, it has recently been demonstrated that the intracellular, or kinase, domain of the EGF receptor has extensive sequence homology with the transforming gene product of v-erb-B, which is also structurally related to v-src, but which has not yet been demonstrated to be a kinase (9, 10).

Structurally, the EGF receptor is an intrinsic membrane glycoprotein with an apparent molecular weight of 170-175 kDa (11, 12). It contains a core polypeptide of 1186 amino acids (154 kDa) and an apparent 36-41 kDa of N-linked carbohydrate. The entire cDNA and corresponding amino acid sequence has recently been reported (10). Studies on the biosynthesis of the receptor in A431 cells using pulse-chase techniques have shown that the initial translation product containing core oligosaccharide chains has an apparent molecular mass of 160 kDa and that this is further processed to the mature receptor by capping with terminal sugars (12). A 95-105-kDa receptor-related protein consisting of the extracellular domain has also been observed (12, 13) and Northern blot analysis has confirmed the existence of multiple mRNAs for the receptor in A431 cells, but not normal human fibroblasts (10, 14).

We have examined the post-translational processing of the EGF receptor which is responsible for the delayed acquisition of EGF-binding activity by using an affinity matrix to adsorb active receptor from extracts of pulse-labeled A431 cells. It is expected that an understanding of the mechanism by which the receptor acquires EGF-binding activity will provide information on the relationship of its structure and biological function.

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EXPERIMENTAL PROCEDURES

Materials—EGF was purified from mouse submaxillary glands (Pel-Freez) by the method of Savage and Cohen (15). Affi-Gel 10 was purchased from Bio-Rad and EGF-Affi-Gel was prepared as described by Cohen et al. (16). Approximately 1.5 mg of EGF were coupled/ml of packed resin. Protein A-Sepharose was purchased from Pharmacia and tunicamycin was obtained from Calbiochem-Behring. L-[14C] Methylamine (1100-1400 Ci/mmol) and Na2115I (>14 mCi/µg of iodine) were purchased from Amersham. EGF was iodinated by the lactoperoxidase method employing Enzymobeads from Bio-Rad. Trypsyl (10,000 Kallikrein units/ml) was purchased from FBA Pharmaceuti- cals and pepstatin, leupeptin, antipain, and chymostatin were ob- tained from Penninsula Laboratories. Benzamidine, concanavalin A, and PMSF were purchased from Sigma. Protease inhibitor mixtures I and II were prepared from the above as described by Ronnet al et. (17).

Cell Culture—A431 Human epidermoid carcinoma cells were ob- tained from Dr. George Todaro (Oncogen, Seattle, WA) and main- tained in DMEM containing 10% FBS in an atmosphere of 10% CO2 at 37 °C. Cell number was determined with a Coulter ZBI cell counter after trypsin treatment.

EGF Receptor Purification—Human EGF receptor was affinity purified from A431 cells by a modification of the procedure of Cohen (16). Forty 10-cm dishes of A431 cells (approximately 2.5 x 107 cells/dish) were scraped into 50 ml of scurf buffer (20 mM HEPES, pH 7.4, 5 mM EDTA, 1 mM PMSF, 1% Trypsyl, and 1 µl/ml of protease inhibitor mixtures I and II) at 4 °C. The cells were homogenized with 20 strokes in a glass homogenizer with a Teflon pestle and the suspension was centrifuged at 190,000 x g for 45 min. The pellet was rehomogenized in 10 ml of extraction buffer (20 mM HEPES, pH 7.4, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM

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*This work was supported by Research Grant from the American Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Postdoctoral Fellow- ship AM-07319.
PMSE, 1% Trasylol, and 1 μl/ml of protease inhibitor mixtures I and II at 4°C, and after centrifugation as before, the supernatant was applied to an EGF Affi-Gel column (0.9 x 6 cm, prepared as described above) equilibrated in 20 mM HEPES, pH 7.4, 0.1% Triton X-100. The flow-through volume was reapplied and then the column was washed with 30 ml of the same buffer. The receptor was eluted in 20 mM NH₄OH, pH 9.8, 0.1% Triton X-100 and neutralized with 1 M HEPES, pH 7.4. Fractions were assayed for EGF binding activity as described by Krupp et al. (18) and pooled fractions were concentrated to 1.7 ml on an Amicon FM-30 membrane with quantitative recovery of binding activity. Typically, 200-400 μg of protein were recovered as determined by the method of Peterson (19).

Antibody Production—Approximately 50 μg of receptor was further purified on a 6% Laemmli polyacrylamide slab gel in the presence of SDS but under nonreducing conditions. The receptor was visualized by Coomassie Blue staining of a narrow strip cut from the center of the slab, and the excised gel band was homogenized in 2 ml of Freund's complete adjuvant for the initial immunization of the rabbit. This was followed by a similar boost (using incomplete adjuvant) at 2 weeks and the first bleed 5 weeks later. Boosts were repeated after one and three months, and the serum used for these experiments was obtained two weeks after the last boost. An IgG fraction was prepared from the serum by elution from protein A-Sepharose with 0.1 M glycine, pH 2.8, followed by neutralization and overnight dialysis against 20 mM HEPES, pH 7.5, 5% glycerol.

Metabolic Labeling of A431 Cells with [35S]Methionine—A431 cell monolayers were cultured on 60-mm dishes and were used at a cell density of approximately 8 x 10⁵ cells/dish. Pulse-chase experiments were performed by washing the monolayers twice with PBS and incubating at 37°C with 2 ml of methionine-free DMEM supplemented with 1 μCi/ml unlabeled methionine, 10% dialyzed FBS, and 0.15 mM [35S]methionine. After 15 m pulse medium was removed, the monolayers were washed once with PBS, and 3 ml of chase medium consisting of normal DMEM and 10% FBS were added for the indicated times. At the end of each chase, the cells were harvested by removing the chase medium, washing with 3 ml of cold PBS and 3 ml of scrape buffer and scraping into 1 ml of buffer. Centrifugation and extraction of the membrane pellet was performed. The extraction buffer was as described for the receptor purification. For experiments employing tunicamycin, cell monolayers were preincubated with 1 μg/ml of tunicamycin for 4 h and this concentration was maintained during the pulse and chase. Measurement of [35S]methionine incorporation into trichloroacetic acid-precipitable radioactivity indicated 40-50% inhibition of protein synthesis during this period.

Immunoprecipitation and EGF Affi-Gel Adsorption—Typically, 50 μl of the membrane extract (approximately 350,000-400,000 cpm of [35S] and representing 5 x 10⁵ cells) were added to 400 μl of 20 mM HEPES, pH 7.4, containing 70 μg of protein A-Sepharose and 1 μg of EGF receptor IgG and a 20 μl volume of 0.1 M glycine, pH 2.8. For the preparation of proteins labeled with EGF, the receptor was first eluted from the Affi-Gel column and then adsorbed with EGF. Similarly, EGF Affi-Gel was shown to be in excess over receptor for the corresponding affinity adsorptions.

RESULTS AND DISCUSSION

The biosynthesis of the EGF receptor and its acquisition of ligand-binding activity were studied in A431 cells by labeling with [35S]methionine in a short pulse and then chased for various times in the presence of medium containing unlabeled methionine. Total cellular membranes were isolated and solubilized in 1% Triton X-100, and then the receptor was immunoprecipitated and subjected to SDS-PAGE. Similarly, receptor which was capable of binding EGF was adsorbed from the same cell extract with the affinity matrix EGF Affi-Gel. To demonstrate quantitative immunoprecipitation, 70 μg of polyclonal IgG (the standard amount employed for all immunoprecipitations) were titrated with aliquots of membrane extract representing from 1.1 to 11 x 10⁶ cells (10 to 100 μl of extract). These results (data not shown) indicated a linear relationship between receptor immunoprecipitated and extract volume up to 100 μl, so 50 μl (approximately 5 x 10⁶ cells) were used for all experiments to remain in antibody excess. Similarly, EGF Affi-Gel was shown to be in excess over receptor for the corresponding affinity adsorptions.

Fig. 1 shows the autoradiograms of SDS-polyacrylamide gels of total immunoprecipitations and EGF Affi-Gel adsorptions of extracts of A431 cells from the pulse-chase experiment. As reported previously (12), the immunoprecipitates of the initially labeled EGF receptor species have apparent molecular weights of 160 and 95 kDa. With chase, the 95-kDa peptide is lost by secretion into the medium (13) and the 160-kDa species is further processed into a 175-kDa polypeptide. Fig. 2 shows the equivalent experiment in cells treated with 1 μg/ml of tunicamycin, which inhibits N-linked core oligosaccharide addition. In this case only the 133- and 77-kDa aglycosylated species are observed and they appear to remain unchanged for up to 180 min. The EGF Affi-Gel precipitates reveal two aspects of receptor processing not evident from simple immunoprecipitation. First, neither aglyco-species possesses the capacity to bind EGF. This would suggest that either glycosylation per se is required for ligand binding, or that glycosylation must precede a later processing step where binding activity is acquired. Secondly, the acquisition of binding activity occurs relatively late in the processing pathway. Fig. 1 clearly demonstrates that without a chase the bulk of the 160-kDa immunoprecipitable material does not bind EGF and that full binding activity is not acquired for 120 min.

Also evident in the immunoprecipitations are two polypeptides with apparent molecular weights of 110 and 100 kDa which turn over with a half-time of approximately 20 min. These species do not bind EGF but do contain a small amount of N-linked carbohydrate since treatment with tunicamycin decreases their apparent molecular masses by 3-4 kDa. Since there are apparently at least 11 N-linked oligosaccharide chains on the 160-kDa receptor precursor (12) contributing an apparent molecular mass of 28 kDa, these species would appear to contain 1 or 2 glycosylation sites. The fact that these polypeptides are observed only at the early chase times would suggest that they are not proteolysis products and may represent translation products of multiple receptor mRNAs (10, 14).

These results indicate that the acquisition of EGF binding activity is a complicated post-translational process. Clearly, glycosylation is required since in the presence of tunicamycin neither aglyco-EGF receptor polypeptide (133 or 77 kDa) acquires EGF binding activity. This suggests two possibilities as follows: 1) oligosaccharide chains are intrinsically involved in ligand binding or 2) glycosylation is required before a later activation step can occur. In the latter process, glycosylation could be reviewed as necessary either for an actual post-translational modification whereby activity is acquired, or simply for proper translocation of the precursor to an intracellular site where activation occurs. It is interesting to note that the aglyco-species do not undergo any further processing or apparent degradation for at least 3 h, which would appear to be analogous to the synthesis of the insulin receptor, where in the presence of tunicamycin a 180-kDa aglyco-polypeptide...
Fig. 1. Kinetics of the post-translational acquisition of EGF-binding capacity of the EGF receptor in A431 cells. Cells were incubated with 0.15 mCi/ml of [35S]methionine for 15 min and then chased with medium containing unlabeled methionine for the indicated times. A, [35S]-receptor capable of binding EGF was adsorbed to EGF Affi-Gel from Triton X-100 extracts of total cellular membranes; B, [35S]-receptor was immunoprecipitated from the same volume of extract with anti-EGF receptor IgG. [35S]-labeled receptor proteins isolated by these procedures were separated by SDS-PAGE on gels containing 7.7% acrylamide. Molecular weight markers are: myosin, 205,000; /-galactosidase, 116,000; phosphorylase b, 97,000; bovine serum albumin, 66,000, and ovalbumin, 45,000.

Fig. 2. The effect of tunicamycin on the biosynthesis and ligand-binding activity of the EGF receptor in A431 cells. Cell monolayers were preincubated with 1 µg/ml of tunicamycin for 4 h. The pulse with [35S]methionine and chase were performed in the presence of 1 µg/ml of tunicamycin as described in Fig. 1. Chase times are as indicated. Triton X-100 cell extracts were treated with EGF Affi-Gel and anti-EGF receptor IgG to isolate receptor capable of binding EGF and immunoprecipitable receptor, respectively. E, EGF Affi-Gel; I, immunoprecipitation; PI, preimmune serum. Proteins were separated by SDS-PAGE employing a linear gradient from 4 to 15% acrylamide. Molecular weight standards are as described in Fig. 1 with the addition of carbonic anhydrase (29,000).

accumulates without being further processed and does not reach the cell surface (17). Since both the 160- and 175-kDa receptor species bind EGF (see Fig. 1), it would appear that the conversion of high-mannose to complex carbohydrate moieties is not required for binding activity and therefore that the activation occurs prior to exit from the Golgi or passage through the trans Golgi.

Concerning the nature of this activation, a number of possible covalent modifications must be considered. Proteolytic cleavage of a proreceptor can be eliminated on the basis of the cDNA sequence (10), which precludes the loss of any fragment larger than the 24-amino acid signal peptide, as well as the fact that the molecular weight of the receptor does not change significantly during activation. Although both the acetylcholine (21) and transferrin (22) receptors, as well as the transforming proteins of Rous sarcoma virus, Harvey sarcoma virus, and Abelson virus (23), contain covalent, post-translationally added fatty acyl groups, the native EGF receptor apparently does not (23). Phosphorylation, perhaps mediated by another protein kinase, is an intriguing possibility which we have not yet investigated. Another possibility, in the light of the recently published amino acid sequence, is the formation of disulfide bonds. Ullrich et al. (10) have shown that the extracellular region of the receptor contains 51 cysteines (out of a total of 60) located in two domains of approximately 170 amino acids each. Although it is not yet known how many of these residues are present as disulfides, it would be reasonable to expect that extensive disulfide linkages would be required to define a ligand binding site, particularly in an extracellular domain. We are presently investigating the exact
nature of this post-translational modification.

Although the functional significance of glycosylation of the EGF receptor is not known, it is clear that it is necessary for the acquisition of ligand binding activity. Whether this is due to a contribution to the proper intracellular translocation of intermediates in the processing pathway of the receptor or to the tertiary structure of the peptide necessary for further modification, such as disulfide-bond formation, remains unclear. A similar requirement of N-linked glycosylation and a modification, such as disulfide-bond formation, remains uncles.

60-90-min lag time for the acquisition of binding activity has been demonstrated for the insulin receptor (24-26). It is interesting to note that human fibroblast low-density lipoprotein receptors synthesized in the presence of tunicamycin still form which is unaltered from the normal species in terms of nature of this post-translational modification.

REFERENCES