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Aspects of the Metabolism of the Epidermal Growth Factor Receptor in A431 Human Epidermoid Carcinoma Cells

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The biosynthesis and posttranslational metabolism of the epidermal growth factor (EGF) receptor were examined in the A431 human epidermoid carcinoma cell line. Polyclonal antibody against the receptor specifically immunoprecipitated two [^{35}S]methionine-labeled proteins of M_r = 160,000 and 170,000. Pulse chase experiments showed the M_r = 160,000 protein to be a precursor of the M_r = 170,000 protein. Preincubation with tunicamycin resulted in immunoprecipitation of a single band of M_r = 130,000, whereas monensin inhibited maturation to the M_r = 170,000 form. Digestion of the M_r = 160,000 and 170,000 proteins with endoglycosidase H resulted in the appearance of M_r = 130,000 and 165,000 proteins, respectively. Prolonged pulse-chase experiments indicated that the half-life of the receptor is ca. 20 h in the absence of EGF and 5 h in the presence of EGF. Approximately three- to five-fold more phosphate is incorporated into the mature receptor upon addition of EGF, due primarily to increases in levels of phosphotyrosine and phosphoserine. Phosphate was also present on the M_r = 160,000 protein and the M_r = 130,000 protein found in the presence of tunicamycin.

The epidermal growth factor (EGF) receptor is one of a diverse group of plasma membrane proteins involved in the binding and internalization of extracellular macromolecules. This group includes the receptors for high-density lipoprotein, insulin, transferrin, mannose 6-phosphate, and asialoglycoproteins (3). The mature EGF receptor is an M_r = 170,000 glycoprotein consisting of a single polypeptide chain (7), and interaction of EGF with the receptor initiates a complex sequence of events ultimately resulting in stimulation of DNA synthesis and cell division in target cells (1, 4). An early consequence of EGF binding is the activation of a tyrosine-specific protein kinase which appears to be integrally associated with the receptor (5, 6). EGF binding to the receptor also causes internalization of ligand-receptor complexes via endocytosis (12, 24). The role of these phenomena in EGF-induced cell proliferation is unknown.

Studies of the synthesis and posttranslational processing of free and ligand-bound receptors should contribute to the understanding of the mechanisms by which EGF acts as a mitogen. In this report aspects of metabolism of the EGF receptor in A431 epidermoid carcinoma cells are examined through the use of polyclonal antibody raised against the EGF receptor.

MATERIALS AND METHODS

Cell culture. A431 human epidermoid carcinoma cells (generously provided by J. DeLarco, National Institutes of Health) were grown in Dulbecco modified Eagle medium (DME) containing 5% calf serum (Flow Laboratories, Inc.) and 50 μg of gentamicin per ml.

Materials. Receptor-grade EGF was from Collaborative Research, Inc., monensin was from Calbiochem-Behring, endoglycosidase H was from Miles Laboratories, Inc., and tunicamycin and protein A-agarose were from Sigma Chemical Co. L-[^{35}S]methionine (1,000 Ci/mmol) was purchased from New England Nuclear Corp., [γ-^{32}P]ATP (3,000 Ci/mmol) was from Amersham Corp., and carrier-free ^{32}P, was from ICN Pharmaceuticals, Inc. Mouse monoclonal antibody against the EGF receptor from A431 cells was a gift from B. Ozanne, University of Texas at Dallas.

Radioisotope labeling. For all experiments A431 cells were grown in multiwell plates (2.2 cm^2 per well, BD Labware). Labeling of cells (4×10^4 to 6×10^4 per well) with [^{35}S]methionine (100 μCi/ml) was carried out in methionine-free DME plus 5% dialyzed calf serum (0.25-ml total volume) for various times as specified in the legends to Fig. 1, 3, 4, and 5. For pulse-chase experiments cells were labeled for the times indicated in Fig. 3 and 4 and then washed and incubated with normal DME containing 5% calf serum. Labeling with ^{32}P was performed as previously described (8).

Monensin treatment was according to Jacobs et al. (15), tunicamycin treatment was according to Omary and Trowbridge (21), and endoglycosidase H digestion was according to Siuta-Mangano et al. (28).

Immunoprecipitation. Polyclonal antiserum against the sodium dodecyl sulfate (SDS)-denatured EGF receptor (S. Decker, Arch. Biochem. Biophys., in press) was used for most immunoprecipitation experiments. Labeled cells were lysed in 50 μl of hot 2% SDS, heated at 100°C for 5 min, diluted 1:20 with RIPA buffer (16) without SDS, and centrifuged. Extracts were mixed with 2 μl of antiserum and incubated at 4°C for 30 min, and 30 μl of 1:1 (vol/vol) RIPA buffer–protein A-agarose was added for another 30 min. The agarose beads were washed three times with 1 ml of RIPA buffer and once with 50 mM Tris-hydrochloride, pH 7.4, and then heated at 100°C for 5 min in electrophoresis sample buffer (19). When mouse monoclonal antibody against the EGF receptor was used for immunoprecipitation, cells were lysed in RIPA buffer, and 3.6 μg of immunoglobulin G was used for each sample.

In vitro phosphorylation of immunoprecipitated EGF receptor. Approximately 10^5 A431 cells were washed once with phosphate-buffered saline and extracted with a solution of 1% Triton X-100, 10% glycerol, and 10 μg of aprotinin per ml in phosphate-buffered saline (TG buffer) (26). The lysate was cleared by centrifugation (2 min in an Eppendorf model 3200 centrifuge), and 2 μl of rabbit anti-EGF receptor serum was added to the supernatant. After 30 min at 0°C, 30 μl of 1:1 protein A-agarose beads–TG buffer was added, and samples were incubated with shaking at 4°C for 30 min. The beads were washed three times with 1 ml of TG buffer and

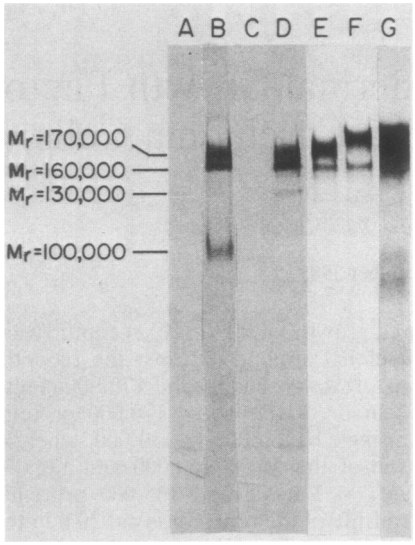

FIG. 1. Immunoprecipitation of the EGF receptor from A431 cells. For lanes A to D, cells were labeled with [^{35}S]methionine for 6 h as described in the text. Lysates from 4×10^4 to 6×10^4 cells were immunoprecipitated with 3.6 μg of control murine immunoglobulin G (A), 3.6 μg of anti-EGF receptor monoclonal antibody (B), 2 μg of preimmune rabbit serum (C), and 2 μl of rabbit anti-EGF receptor serum (D). For lanes E and F, cells were labeled with [^{35}S]methionine for 12 h and incubated in the absence (E) or presence (F) of 100 ng of EGF per ml during the final hour of labeling. In vitro phosphorylation of immunoprecipitated EGF receptor (performed as described in the text) is shown in lane G. Positions of the M_r = 100,000, 130,000, 160,000, and 170,000 regions of the gels are indicated. Samples were run on 6.5% SDS-polyacrylamide gels.

suspended in 50 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–1 mM $MnCl_2$, pH 7.4. Five micromolar [γ-^{32}P]ATP (200 Ci/mmol) was added for 5 min at 0°C, and 50 μl of electrophoresis sample buffer was added.

Miscellaneous methods. Analysis of phosphoamino acids, partial V8 protease mapping, preparation of SDS-polyacrylamide gels, and determination of protein were performed as described previously (8).

RESULTS

Various aspects of EGF receptor metabolism were examined in experiments involving immunoprecipitation with rabbit antiserum against the EGF receptor (Decker, in press). Several lines of evidence suggest that the antiserum does indeed recognize the EGF receptor.

(i) The rabbit antiserum specifically immunoprecipitated two proteins of M_r = 160,000 and 170,000 from [^{35}S]methionine-labeled A431 cells, as did a monoclonal antibody against the EGF receptor (Fig. 1B and D). A third protein of M_r = 130,000 was recognized by the rabbit antibody, and the monoclonal antibody also immunoprecipitated an M_r = 100,000 protein. V8 protease treatment of the M_r = 160,000 and 170,000 proteins immunoprecipitated with the two different antibodies produced identical peptide maps for all four proteins, whereas the M_r = 100,000 protein immunoprecipitated by the monoclonal antibody was unrelated to the M_r = 160,000 and 170,000 proteins (Fig. 2; Decker, in press). However, the V8 protease map of the M_r = 100,000 protein was very similar to that of the M_r = 130,000 protein recognized by the rabbit antiserum.

(ii) EGF treatment of cells resulted in increased incorporation of ^{32}P into the M_r = 170,000 protein, which may have caused the apparent increase in the molecular weight of the [^{35}S]methionine- and ^{32}P-labeled M_r = 170,000 proteins from M_r = 170,000 to 175,000 (Fig. 1E and F and 5A and B, respectively). A similar apparent molecular weight increase has been reported for in vitro-phosphorylated EGF receptor labeled in the presence of EGF (10).

(iii) When immunoprecipitates from A431 cells were incubated with [^{32}P]ATP, an M_r = 170,000 protein became heavily labeled (Fig. 1G) exclusively on tyrosine residues (data not shown). The association of an in vitro cyclic nucleotide-independent, tyrosine-specific protein kinase activity with the EGF receptor has been well documented (5, 6).

In A431 cells pulsed with [^{35}S]methionine for 45 min, only the M_r = 160,000 protein was immunoprecipitated (Fig. 3). The chase into the M_r = 170,000 protein began after 1 to 2 h, and conversion was complete at ca. 20 h. Similar kinetics of loss of the M_r = 160,000 band occurred in the absence or presence of EGF (data not shown).

The half-life of the mature EGF receptor in the absence and presence of EGF was calculated from the data in Fig. 4 and found to be about 20 h in the absence of EGF and 5 h in the presence of EGF. Protein turnover in general was not affected by EGF addition, as judged by comparing levels of [^{35}S]methionine-labeled proteins in whole-cell extracts before and after a 24-h chase with medium containing unlabeled methionine (Fig. 4C). The half-lives of the cytoskeletal protein vinculin and of the M_r = 34,000 protein thought to be a substrate for the Rous sarcoma virus *src* gene product (22) were specifically examined and found to be unaffected by the EGF addition (data not shown). The rate of synthesis of the EGF receptor did not appear to be altered by EGF addition, as judged by the rates of incorporation of [^{35}S]methionine into the receptor in the absence and presence of EGF (data now shown).

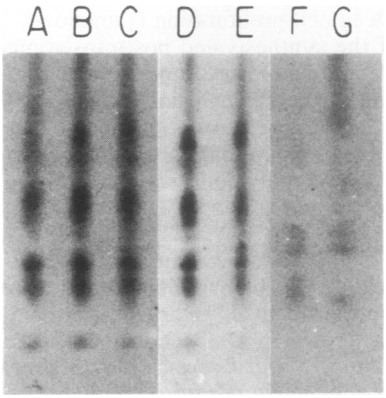

FIG. 2. Partial V8 protease maps of immunoprecipitated proteins. Proteins were eluted from gels and treated with V8 protease (2 μg per sample) as previously described (8). Cleavage maps of the M_r = 160,000 (A) and 170,000 (B) proteins immunoprecipitated by monoclonal antibody against the EGF receptor (as shown in Fig. 1B), of the M_r = 160,000 (C) and 170,000 (D) proteins immunoprecipitated by polyclonal anti-EGF receptor serum (as shown in Fig. 1D), of the M_r = 130,000 protein immunoprecipitated from tunicamycin-treated cells (as shown in Fig. 5D), of the M_r = 130,000 protein immunoprecipitated by the polyclonal antiserum (as shown in Fig. 1D), and of the M_r = 100,000 protein immunoprecipitated by the monoclonal antibody (as shown in Fig. 1B) are shown.

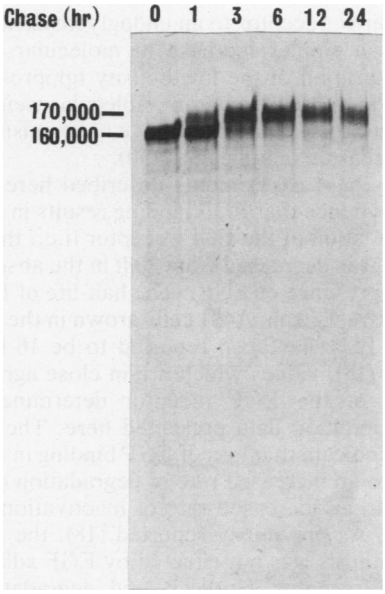

FIG. 3. Conversion of the EGF receptor from the immature M_r = 160,000 form to the mature M_r = 170,000 form. A431 cells (4×10^4 cells per well) were labeled for 45 min with 100 μCi of [^{35}S]methionine per ml as described in the text. Cells were then washed once with 1 ml of normal DME and incubated for various times in 1 ml of normal DME with 5% calf serum. Lysates were immunoprecipitated with saturating amounts of rabbit anti-EGF receptor antiserum (2 μl) at the times indicated, and 6.5% SDS-polyacrylamide gels were prepared as described in the text. The M_r = 160,000 and 170,000 regions of the gel are indicated.

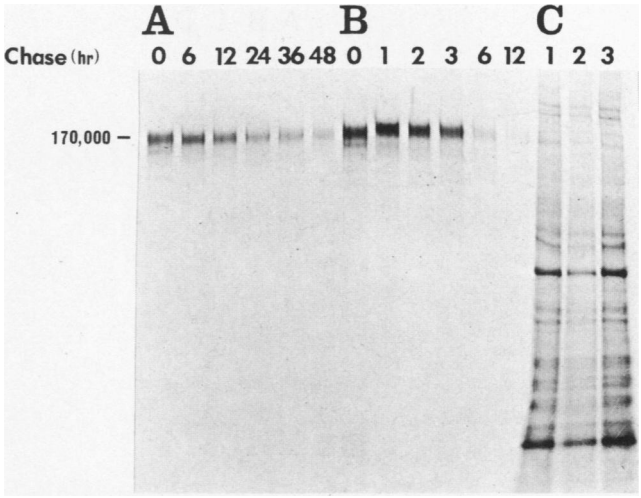

FIG. 4. Turnover of the EGF receptor from A431 cells in the absence (A) or presence (B) of EGF. A431 cells (4×10^4 cells per well) were labeled with [^{35}S]methionine as described in the text. After 12 h of labeling, cells were washed once with 1.0 ml of normal DME and incubated for various times as indicated with normal DME containing 5% calf serum. For (B), 100 ng of EGF per ml was included throughout the chase. Cell lysates were immunoprecipitated and SDS-polyacrylamide gels were prepared as for Fig. 3. The M_r = 170,000 region of the gel is indicated. In (C), cells were labeled with [^{35}S]methionine for 12 h and lysed directly into gel sample buffer (1), washed and incubated in normal DME plus 5% calf serum for 24 h before lysis (2), or washed and incubated in DME plus 5% calf serum containing 100 ng of EGF per ml for 24 h before lysis (3).

Since the EGF receptor has been previously shown to be a glycoprotein (6), it was believed that the transition from the M_r = 160,000 protein to the M_r 170,000 form might involve glycosylation. Experiments in which the carboxylic ionophore monensin (known to inhibit terminal glycosylation of newly synthesized proteins in the Golgi complex [29]) was used supported this conclusion. In the presence of monensin, further processing of the M_r = 160,000 protein to the M_r = 170,000 mature form was blocked (Fig. 5A and B). This result also raised the possibility that the immature M_r = 160,000 protein contained primarily "high-mannose" asparagine-linked carbohydrate susceptible to digestion with endoglycosidase H, analogous to many other eucaryotic glycoproteins (13). Treatment of immunoprecipitates with endoglycosidase H resulted in the appearance of an M_r = 130,000 protein derived from the M_r = 160,000 protein (Fig. 5E and F). Treatment of the M_r = 170,000 protein with endoglycosidase H caused a decrease in apparent molecular weight to M_r = 165,000 (Fig. 5G and H). This suggests that some high-mannose oligosaccaride is still present in the mature receptor, even though a significant proportion has matured to endoglycosidase H-resistant complex carbohydrate.

In cells treated with tunicamycin, which inhibits asparagine-linked glycosylation (20), only a single band of M_r = 130,000 was immunoprecipitated (Fig. 5C and D). The V8 protease map of this protein was identical to those of the M_r = 160,000 and 170,000 forms of the EGF receptor (Fig. 2E).

Neither the M_r = 160,000 protein in monensin-treated cells nor the M_r = 130,000 protein in tunicamycin-treated cells chased into other proteins when respective inhibitors were removed (data not shown). This may have been due to prior transport of these immature forms of the receptor to intracellular compartments where appropriate processing cannot be carried out (29).

Phosphorylation of EGF receptor. EGF receptor from

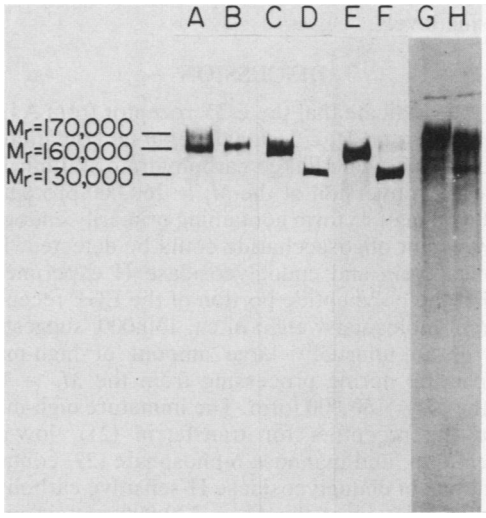

FIG. 5. Effect of monensin, tunicamycin, and endoglycosidase H treatment on the EGF receptor. A431 cells were labeled with [^{35}S]methionine for 4 h (A to E) or 16 h (G and H) as described in the text. One hour before labeling, cultures received 0.1% ethanol (A and C), 0.5 μM monensin (B), or 5 μg of tunicamycin per ml (D), and these additions were maintained throughout the labeling procedure. Control (E and G) and endoglycosidase H-treated (F and H) immunoprecipitates of [^{35}S]methionine-labeled EGF receptor are shown. Endoglycosidase H treatment was as described in the text. EGF receptor was immunoprecipitated as for Fig. 1.

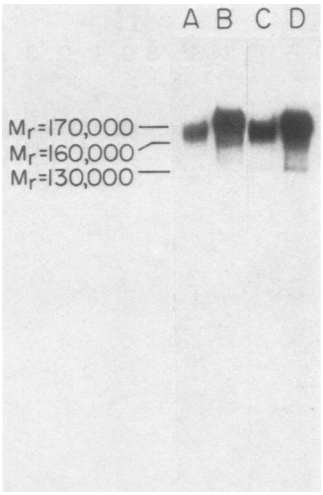

FIG. 6. Phosphorylation of the EGF receptor from A431 cells. Cells were labeled for 12 h with $^{32}P_i$ as described in the text. EGF (100 ng/ml) was added 1 h before immunoprecipitation (B and D), and 5 μg of tunicamycin per ml was added 4 h before immunoprecipitation (C and D).

EGF-treated cells contained a three- to fivefold increase in ^{32}P compared with control cells (Fig. 6). Most of the ^{32}P was incorporated into the M_r = 170,000 mature protein, although a faint ^{32}P-labeled band was present at M_r = 160,000. The M_r = 130,000 protein found in tunicamycin-treated cells also appeared to be phosphorylated. The EGF-induced increase in receptor-bound phosphate was due primarily to increased phosphorylation of serine and tyrosine residues; the amount of phosphothreonine remained relatively constant (Table 1).

For phosphorylation experiments, it was important to denature extracts in hot 2% SDS before immunoprecipitation, since immunoprecipitation of extracts lysed in nondenaturing detergent resulted in much lower estimates of phosphotyrosine levels.

DISCUSSION

These data indicate that the EGF receptor from A431 cells is synthesized as an M_r = 160,000 precursor containing high-mannose *N*-asparagine-linked carbohydrate. Within 1 to 2 h of synthesis, conversion of the M_r = 160,000 protein to the M_r = 170,000 mature form containing primarily endoglycosidase H-resistant oligosaccharide could be detected. Results from tunicamycin and endoglycosidase H experiments revealed that the polypeptide portion of the EGF receptor has an apparent molecular weight of ca. 130,000, suggesting the addition of an unusually large amount of high-mannose oligosaccharide during processing from the M_r = 130,000 form to the M_r = 160,000 form. The immature high-mannose forms of the receptors for transferrin (21), low-density lipoprotein (30), and mannose 6-phosphate (23) contain 8 to 12 kilodaltons of endoglycosidase H-sensitive carbohydrate. The *N*-glycosylation of the M_r = 130,000 protein probably occurs cotranslationally or very soon after translation, since no M_r = 130,000 form was immunoprecipitated without tunicamycin treatment. Cotranslational glycosylation has been demonstrated for several proteins, including ovalbumin (17) and mouse immunoglobulin G heavy chain (22).

Monensin-sensitive, endoglycosidase H-resistant terminal glycosylation of the EGF receptor from A431 cells was detectable within 1 h of synthesis and resulted in a further increase in apparent molecular weight of 10,000. Maturation of the transferrin receptor to an endoglycosidase H-resistant form causes a similar increase in molecular weight (21), whereas maturation of the low-density lipoprotein receptor results in an increase in apparent molecular weight of 40,000 and may occur through an alternative mechanism not involving oligosaccharide modification (30).

The pulse-chase experiments described here provide the first direct evidence that EGF binding results in an increased rate of degradation of the EGF receptor (i.e., the half-life of the receptor was decreased from 20 h in the absence of EGF to 5 h in the presence of EGF). The half-life of EGF-binding activity in extracts from A431 cells grown in the absence and presence of EGF has been reported to be 16 h and 4.5 h, respectively (18), values which are in close agreement with the half-life of the EGF receptor determined from the immunoprecipitation data presented here. The results presented here indicate that loss of EGF binding in EGF-treated cells is due to an increased rate of degradation of receptors, rather than to an increased rate of inactivation of receptor binding (18). As previously reported (18), the rate of EGF receptor synthesis was not effected by EGF addition. These dynamics of receptor synthesis and degradation may be responsible for the decreased EGF binding to the cell surface after EGF addition (i.e., "down-regulation") found in A431 cells (31) and many other cell types (4).

Since EGF receptors which have bound EGF are rapidly internalized and selectively degraded, these processes may play a role in EGF-induced stimulation of DNA synthesis. In support of this conclusion, studies with cyanogen bromide-treated EGF (27) and monoclonal antibodies directed against the EGF receptor (26) indicate that internalization of ligand-receptor complexes is necessary for EGF to exert its mitogenic effects.

Phosphorylation of the EGF receptor also appears to be a significant aspect of receptor metabolism. The EGF receptor from a variety of cell types becomes phosphorylated on tyrosine residues in vitro (5, 8, 9), and analysis of in vivo-phosphorylated receptor from A431 cells reveals the presence of phosphotyrosine, phosphothreonine, and phosphoserine (14). The three- to fivefold EGF-induced increase in receptor-bound ^{32}P reported in this study is somewhat higher then the twofold increase reported by Hunter and Cooper (14). This difference may be due to differences in the cell lysis procedures. The overall increase in receptor phosphorylation was mostly due to higher levels of phosphotyrosine and phosphoserine. The elevated amount of phosphotyrosine may be due to autophosphorylation of receptor molecules, since EGF stimulates a receptor-associated tyrosine-specific protein kinase (5, 6). The nature of the kinases involved in phosphorylation of the serine and threonine residues is not known.

TABLE 1. Levels of acid-stable phosphoamino acids in EGF receptors from control and EGF-treated A431 cells[a]

Phosphoamino acid	Control		+ 100 ng of EGF per ml	
	cpm	%	cpm	%
Phosphotyrosine	290	26.7	1,765	36.3
Phosphothreonine	376	34.6	742	15.3
Phosphoserine	421	38.7	2,351	48.4

[a] Cells were labeled with ^{32}P, EGF receptor was immunoprecipitated and run on SDS-polyacrylamide gels, and phosphoamino acids were analyzed as described in the text. Labeling with ^{32}P was for 15 h, and EGF was added 1 h before lysis.

Most of the phosphate was present on the M_r = 170,000 mature form of the EGF receptor; however, the M_r = 160,000 precursor and the M_r = 130,000 protein found in the presence of tunicamycin were also phosphorylated. Phosphorylation of the M_r = 130,000 protein in the presence of EGF did not result in a shift in molecular weight as is found for the M_r = 170,000 form of the EGF receptor. Only the mature forms of the receptors for transferrin (25) and mannose 6-phosphate (23) were found to be phosphorylated. The significance of phosphorylation of the immature forms of the EGF receptor is not known.

In this study, several aspects of the metabolism of the EGF receptor from A431 epidermoid carcinoma cells were examined with polyclonal antibody raised against the receptor. A431 cells are somewhat unusual in many of their responses to EGF (11), and the general validity of the results presented here must be established with normal cell types.

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