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THE EGF RECEPTOR-KINASE HAS MULTIPLE PHOSPHORYLATION SITES

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Summary: The apparent molecular weight in sodium dodecyl sulfate polyacrylamide gel electrophoresis of epidermal growth factor (EGF) receptor-kinase labeled with 15 μM [γ-^{32}P] ATP increased as much as 10K daltons when EGF was present during labeling. The EGF receptor-kinase phosphorylated at very low ATP concentrations could be shifted to higher molecular weight by adding excess unlabeled ATP both in the presence and absence of added EGF. Tryptic peptide maps made from the EGF receptor-kinase phosphorylated at low and high ATP concentrations showed four major phosphorylated peptides. The presence of 4 major phosphorylated tryptic peptides and the dependency of the molecular weight shift on the amount of phosphate incorporated suggested that multiple phosphorylation sites are present on the receptor-kinase.

Introduction: The mitogenic polypeptide EGF enhances cell proliferation in vivo and in cell culture (see reviews (1,2,3,)). Like other polypeptide hormones, EGF forms a complex with plasma membrane receptors and the complex is internalized and the EGF degraded in lysosomes (1,2,3). Neither the signals for enhanced proliferation nor the stages of processing of the complex during which these signals are generated are known. One potential signal is the increased phosphorylation by ATP of both endogenous and exogenous substrates when EGF binds to its receptor. This EGF enhanced kinase is seen in isolated membranes (4,5,6) in solubilized membranes (7) and in affinity purified receptor preparations (7,8) from A-431 epidermoid carcinoma cells. The increased phosphorylation caused by EGF has also been seen in membrane preparations from other cells (6,9,10) and in intact A-431 cells (11,12) as well.

The major endogenous protein in membranes whose phosphorylation is increased by EGF is probably itself both the EGF receptor and the EGF kinase (8). The kinase and phosphorylated portions of the protein are probably transmembrane to the receptor (13). A

Abbreviations: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; mol. wt., molecular weight.

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115K dalton tryptic fragment which contains the EGF binding site does not contain a site phosphorylated by the kinase (13). However, the size of the kinase domain and the location and even the number of the phosphorylated sites has not been determined. This report shows that the electrophoretic mobility in SDS of this 180K dalton EGF receptor-kinase decreased when it was phosphorylated. Since phosphorylation of the already phosphorylated receptor decreased its electrophoretic mobility, the receptor kinase must have multiple sites of phosphorylation.

Materials and Methods: Mouse EGF, which was provided by Dr. Stanley Cohen, Vanderbilt University, was prepared as described previously (14). The A-431 human epithelioid carcinoma cells were provided by Dr. J. De Larco, National Institutes of Health. Dulbecco's modified Eagle's medium, calf serum and penicillin-streptomycin were obtained from Grand Island Biological Company. New England Nuclear supplied the [γ-^{32}P]ATP at a specific activity of 3000 µCi/mmole. SDS-PAGE protein standards for molecular weight determination were purchased from Bio-Rad Laboratories while Eastman chromogram sheets 13255 cellulose without fluorescent indicator were purchased from Eastman Kodak Company. Equine muscle ATP as the disodium salt was obtained from Sigma Chemical Company. Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone came from Millipore Corporation. All other reagents and chemicals were reagent grade and were purchased from common suppliers.

The A-431 cells were grown to confluency in Falcon T-150 tissue culture flasks containing Dulbecco's modified Eagle's medium supplemented with 20mM HEPES 10% calf serum, 5 units/ml penicillin and 5 µg/ml streptomycin. Shed membrane vesicles were prepared from the cells as described by Cohen, Ushiro and Stoscheck (8) with the following modifications. The cells in the flask were washed three times with 20 ml of PBS and then 20 ml of hypotonic solution (1 part PBS:19 parts H_2O) were added for 20 min at room temperature. After decanting this solution, 20 ml of vesiculation buffer was added to the flask which was shaken gently for 20 min at room temperature. Following an incubation at 37°C for 60 min with gentle shaking, the vesiculation buffer containing shed membrane vesicles was removed from the flask, filtered through gauze to remove clumped cells and centrifuged at 30,000 x g for 15 min at 4°C. The vesicle pellet was resuspended in 20mM HEPES, pH = 7.4, centrifuged as above and resuspended in 20mM HEPES, pH = 7.4, to a concentration of 1-3 mg protein per ml and stored frozen at -70°C. Protein was determined according to Bradford (15) using γ-globulin as a standard.

The phosphorylation reaction mixture contained in a final volume of 60 µL: A-431 vesicles (20-40 µg protein); HEPES buffer (20mM, pH = 7.4) $MnCl_2$ (1mM); ATP (0,15, or 150 µM); [γ-^{32}P]ATP(2-20 µCi) bovine serum albumin (3 µg); EGF (0 or 36 ng). EGF was added 10 sec prior to phosphorylation, which was started by adding the mixture of labeled and unlabeled ATP. After 5 minutes at 0°C, the reactions were either stopped or additional unlabeled ATP was added and the reactions were stopped 1 or 2 minutes later. The reactions were terminated by adding 60 µl of 2x concentrated Laemmli sample buffer (16) and heating at 100°C for 3 minutes. One hundred µL of this mixture (equivalent to 50 µL of original phosphorylation reaction mixture) was electrophoresed on 5% polyacrylamide gel slabs (160 x 140 x 1.5 mm) according to Laemmli (16). The gels were fixed, stained with Coomassie Blue, destained, dried and autoradiographed on Dupont Cronex film as described previously (6).

The phosphorylated 180K dalton band was excised from unfixed polyacrylamide gels, digested with trypsin and lyophilized as described by Tuazon, Merrick and Traugh (17) with the following modifications. The slices were washed for 75 minutes each time and were exposed to 8 µg trypsin in 1 ml of buffer at 37°C for 6 hours. Another 12 µg of trypsin was added and the digestion continued for 14 more hours at 37°C. The slice was washed several times with H_2O and the trypsin digest and the washes were lyophilized and relyophilized 4 times. These tryptic digests of the phosphorylated protein were resolved by 2-dimensional electrophoresis and ascending chromatography on thin layer cellulose plates as described by

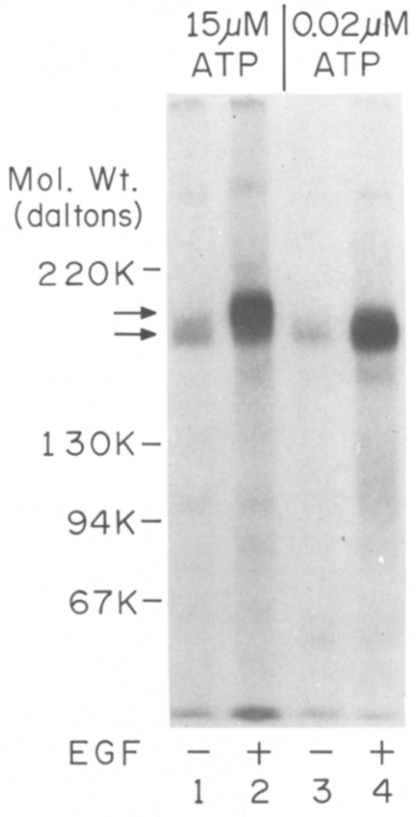

Figure 1: Autoradiograph of electrophoresed A-431 membrane vesicles which were phosphorylated with different concentrations of [γ-^{32}P]ATP in the absence or presence of EGF.

The conditions for phosphorylation, electrophoresis and autoradiography are described under "Materials and Methods." Membrane vesicles at 667 μg/ml were incubated at 0°C for 5 min with [γ-^{32}P]ATP. If present, EGF was added to the vesicles to give a final concentration of 0.6 μg/ml 10 sec before the addition of [γ-^{32}P]ATP. Both 20 μCi/50 μL of 15 μM [γ-^{32}P]ATP (lanes 1,2) and 4 μCi/50 μL of 0.02 μM [γ-^{32}P]ATP (lanes 3,4) were used with (lanes 2 and 4) and without (lanes 1 and 3) EGF. The arrows indicate the position of the major labeled band seen when vesicles are phosphorylated in the presence of EGF with 15 μM (lane 2, upper arrow) or with 0.02 μM (lane 4, lower arrow) [γ-^{32}P]ATP.

Hunter and Sefton (18). Electrophoresis was at 750 volts at 0°C in a TLE 20 thin layer electrophoresis apparatus from Savant Instruments, Inc. until bromophenol blue had migrated 10 cm (about 60 min).

Results and Discussion: The apparent mol. wt. of the major phosphorylated protein labeled with 15 μM [γ-^{32}P] ATP in shed vesicles derived from A-431 cells increased in the presence of EGF by approximately 10K daltons (Fig 1, lanes 1 and 2). The nature of this change in electrophoretic mobility of the EGF receptor-kinase was not clear but could have been due to totally different molecules of similar mol. wt. being phosphorylated in the absence and presence of EGF. Alternatively any of several pre- or post-translational modifications of a

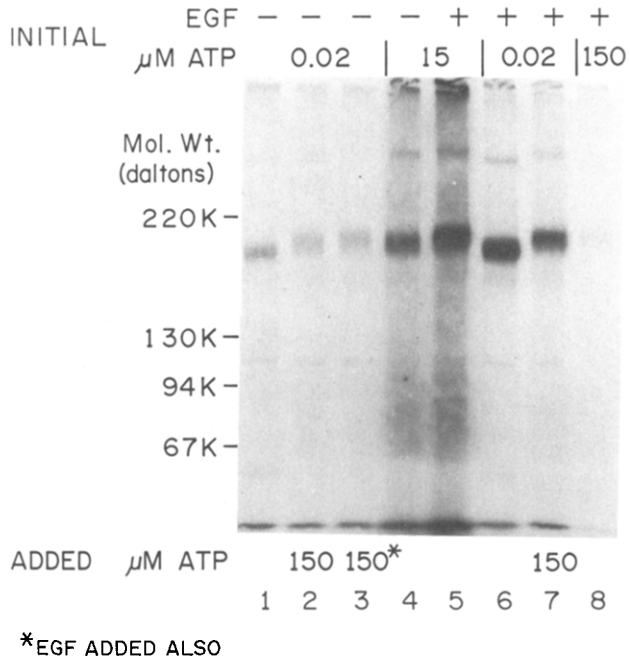

Figure 2: Autoradiograph of electrophoresed A-431 membrane vesicles which had been labeled using [γ-^{32}P]ATP in the absence or presence of EGF and then had excess, cold ATP added.

The conditions for phosphorylation, electrophoresis and autoradiography are described under "Materials and Methods" and in the legend to Figure 1. Vesicles were incubated at 0°C for 5 min with 4 μCi/50 μL of 0.02 μM [γ-^{32}P]ATP (lanes 1,2,3,6,7), with 20 μCi/50 μL of 15 μM [γ-^{32}P]ATP (lanes 4,5) or with 4 μCi/50 μL of 150 μM [γ-^{32}P]ATP (lane 8) in the absence (1,2,3,4) or presence (5,6,7,8) of EGF. After 5 minutes the reactions were terminated (lanes 1,4,5,7,8) or were made 150 μM in ATP (lanes 2,3,7) and 36ng/60 μL in EGF (lane 3) and incubated 2 min more at 0°C before termination. Note the constant mol. wt. of the faint band at 110 K daltons. The faint band above 220 K daltons, whose apparent mol. wt. varies with ATP concentration and whose labeling increases in the presence of EGF, may be a dimer of the EGF receptor-kinase.

single protein could alter its mol. wt. since a modification as small as a single amino acid substitution can change the apparent mol. wt. of a protein as determined by SDS-PAGE (19,20).

To establish if the same molecule was being labeled both in the absence and presence of EGF, we attempted to find conditions where no apparent mol. wt. difference was observed. We found that EGF did not change the mol. wt. of the major phosphorylated substrate if very low concentrations of ATP were incubated with aliquots of the same shed vesicle membrane preparation (Fig. 1, lanes 3,4). Since the same molecule appeared to be phosphorylated in the presence or absence of EGF when very low ATP concentrations were used (mol. wt. = 174 daltons, lower arrow in Fig. 1), the higher mol. wt. species (186K daltons, upper arrow in Fig. 1) phosphorylated at 15 μM ATP in the presence of EGF was likely to be this same molecule.

To determine if the same molecule was being labeled at low and high ATP concentrations in the presence of EGF, we attempted to increase the mol. wt. of the EGF receptor-kinase labeled at low concentrations of [γ-^{32}P]ATP by adding excess unlabeled ATP. Figure 2 (lanes 6,7) shows that adding 150 μM unlabeled ATP to aliquots of EGF receptor-kinase containing membrane vesicles previously labeled at 0.02 μM ATP (4 μCi) caused an increase in the apparent mol. wt. of approximately 12K daltons. Since no significant increase in labeling of the EGF receptor-kinase previously phosphorylated at 0.02 μM ATP would be expected after adding 150 μM unlabeled ATP (no labeling with 4 μCi of 150 μM [γ-^{32}P]ATP in Fig. 2, lane 8), the low and high mol. wt. forms of the major phosphorylated substrate are the same protein molecule. The decrease in label after adding excess unlabeled ATP is probably due to the action of endogenous phosphatases removing radioactive PO_4 as reported previously (5). The action of the endogenous phosphatases may also account for the smaller fold increase in labeling from basal to EGF stimulated levels at high ATP concentration (compare Fig 1, lanes 1,2 with 3,4 and Fig 2, lanes 4,5 with 1,6). Whether or not the action of phosphatases is responsible for the smaller fold increase, it is clear that optimal EGF stimulation of phosphorylation is obtained experimentally at very low ATP concentrations.

Since the observed change in the apparent mol. wt. of one protein, the phosphorylated EGF receptor-kinase, appeared to be dependent on the ATP concentration, the hypothesis that the phosphate incorporated into this entity was the probable cause of the decreased mobility in SDS-PAGE was formulated. At least two other proteins, isozyme II of the cAMP dependent protein kinase (21,22) and protein S6 derived from the 40 S ribosomal subunit (23), show decreased mobility in SDS-PAGE following their phosphorylation. The amount of phosphate incorporated into the EGF receptor-kinase as a function of the ATP concentration was therefore an important question. From the density of autoradiographs or from direct scintillation counting of the appropriate solubilized portion of the dried gel, the labeling of the EGF receptor-kinase was quantitatively comparable in the presence of EGF using either 4 μCi of [γ-^{32}P]ATP at 0.02 μM or 20 μCi of [γ-^{32}P] ATP at 15 μM. Comparing the low to the high ATP concentration, the specific radioactivity was 140 times greater and 5 times as much of the available radioactive phosphate was incorporated. Therefore, only 5/140 = 1/28 as much phosphate was incorporated at 0.02 μM ATP as at 15 μM ATP which supports the hypothesis that increased phosphate incorporation increased the apparent mol. wt.

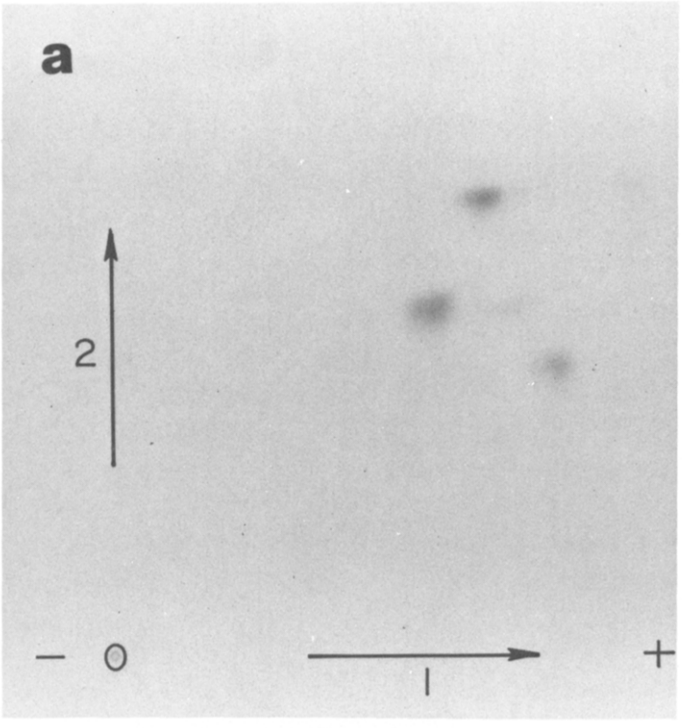

Figure 3: Autoradiographs of two-dimensional tryptic peptide maps of the phosphorylated EGF receptor-kinase.

The procedure for phosphorylation, electrophoresis, trypsin digestion, two-dimensional peptide mapping and autoradiography are described under "Materials and Methods." A-431 membrane vesicles were incubated at 0°C for 5 min in the presence of EGF with [γ-^{32}P] ATP. After 5 minutes the reactions were terminated or made 150 μM in ATP for 1 min at 0°C and then terminated. Following electrophoresis of these reaction mixtures, the major labeled band was cut from the gel and treated with trypsin. Both 3 μCi/50 μL of 0.02 μM [γ-^{32}P] ATP (9a,b) and 15 μCi/50 μL of 15 μM [γ-^{32}P] ATP (c) were used for phosphorylation. One phosphorylation reaction (b) had 150 μM ATP added for the last 1 min before it was terminated. The arrows labeled 1 and 2 indicate the direction of electrophoresis or ascending chromatography respectively. The origin is indicated by 0.

The increased mol. wt. of the receptor-kinase at 15 μM ATP was only obvious when EGF was present during phosphorylation. This effect of EGF, according to the above hypothesis, should have been simply due to the increased phosphate incorporation resulting from the EGF stimulation of its kinase. If this was the basis for the EGF induced mol. wt. increase of its receptor-kinase, then adding excess cold ATP to the receptor labeled in the absence of EGF should also increase its mol. wt. Figure 2 (lanes 1,2) shows that, consistent with this hypothesis, adding excess cold ATP to preparations previously phosphorylated at low ATP concentrations in the absence of EGF induced the change in mobility of the labeled species. Essentially no further increase in mol. wt. was observed by adding EGF along with the excess unlabeled ATP (Fig 2, lane 3).

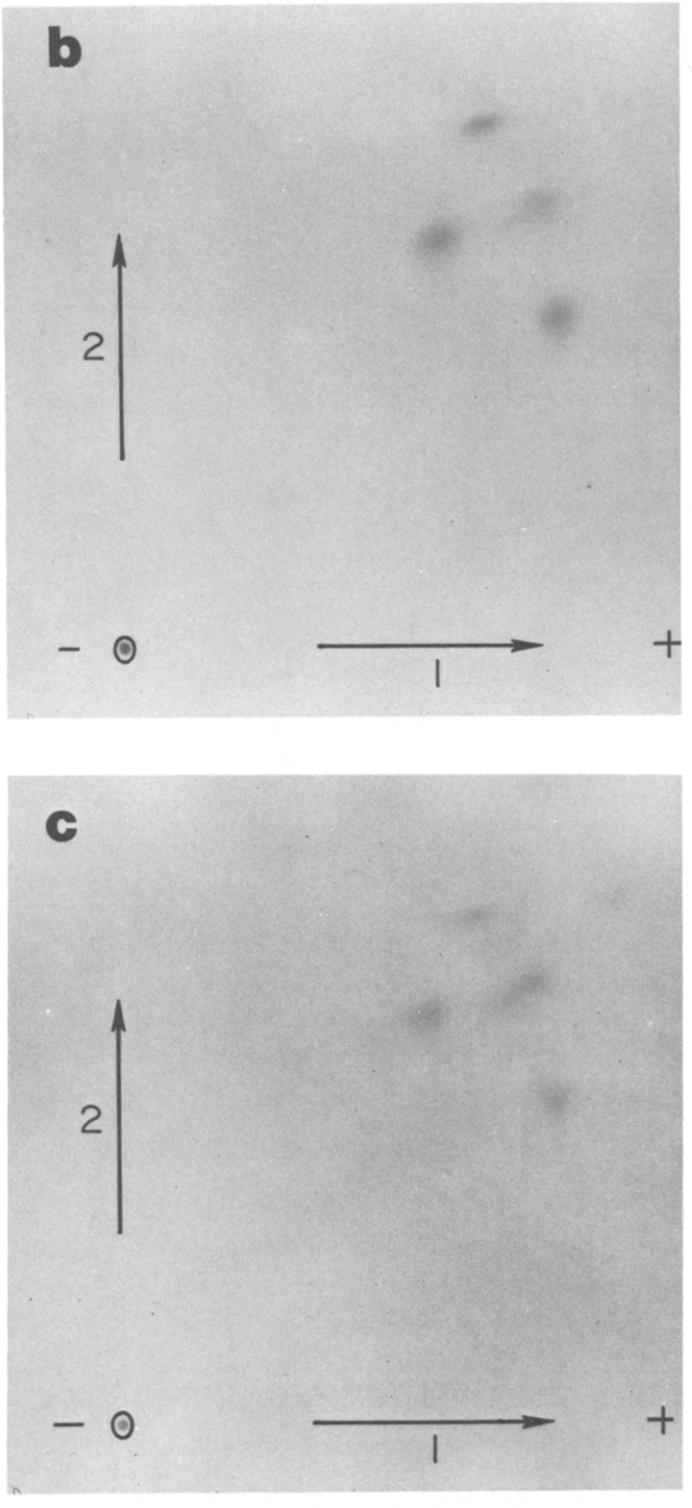

Fig. 3--Continued.

If the increase in apparent mol. wt. of the EGF receptor-kinase depends on the amount of phosphate incorporated, then there must be more than one phosphorylation site per molecule. Multiple phosphorylation sites are required because the 174K dalton form of the receptor (lower arrow in Fig. 1) was labeled and therefore at least singly phosphorylated before more phosphate incorporation could increase its apparent mol. wt. To determine if there were multiple sites of phosphorylation, the phosphorylated species of EGF receptor-kinase were cut from the SDS-polyacrylamide gels and peptide maps prepared. Figure 3 shows the peptide maps prepared from the receptor-kinase labeled under different conditions. With the method used, none of the radioactive spots shown in Fig 3 corresponded to the mobility of either [γ-^{32}P]ATP or $^{32}PO_4$. If trypsin was omitted from the system, no radioactive spots were detected after the 20 hour incubation. There was only a slight difference in the peptide maps derived from the EGF receptor-kinase phosphorylated at 0.02 μM ATP (Fig 3a, 174K dalton species) or at 15 μM ATP (Fig 3c, 186K dalton species). When the 0.02 μM ATP phosphorylated species was subsequently treated with 150 μM unlabeled ATP, its peptide map (Fig. 3b) was virtually identical to the map of the 15 μM ATP phosphorylated species (Fig 3c). Since the appearance of more than one phosphorylated peptide after exhaustive trypsin digestion indicates multiple phosphorylation sites, the increase in apparent mol. wt. of the already phosphorylated EGF receptor-kinase was probably due to more extensive phosphorylation of the receptor. Since the peptide maps were so similar, some features of the effect of phosphorylation on the EGF receptor-kinase were apparent. 1) No unique sites of phosphorylation were present when higher levels of ATP were used suggesting that the cumulative total of phosphates on the EGF receptor-kinase determines its electrophoretic mobility in the SDS-PAGE system used. 2) The order of addition of phosphate to the various phosphorylation sites on the EGF receptor-kinase appeared to be random. Figure 4 depicts this interpretation of the autophosphorylation of the EGF receptor-kinase at low and high ATP concentrations.

The multiple phosphorylation sites of the EGF receptor-kinase indicated by the four major tryptic peptides were probably on tyrosine residues, since only phosphotyrosine was detected with either membranes (24) or shed membrane vesicles (S. Cohen, personal communication) from A-431 cells autophosphorylated using ATP. Hunter and Cooper (11) observed a mobility change in the EGF receptor-kinase after phosphorylation in intact A-431

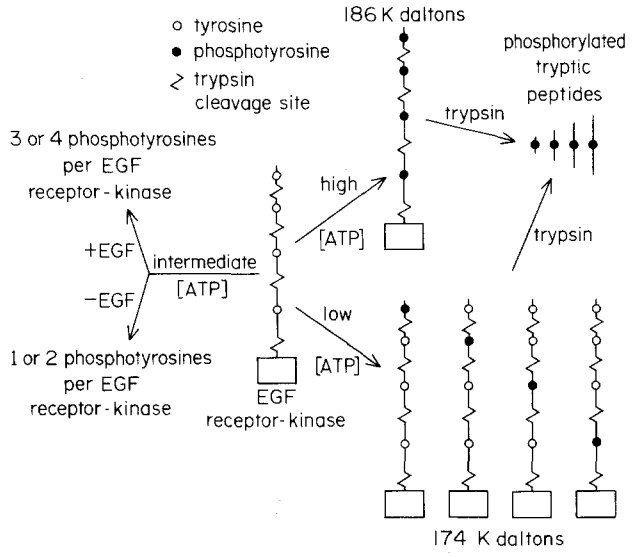

Figure 4: A model of phosphate incorporation into the EGF receptor-kinase to explain the effects of phosphorylation at low, intermediate, or high ATP concentrations on the apparent molecular weights and on the tryptic peptide maps.

cells but found that the molecule contained phosphoserine, phosphothreonine and phosphotyrosine. Reynolds et al (25) prepared tryptic peptide maps of the EGF receptor-kinase phosphorylated in detergent solubilized A-431 cells and found one major and seven minor phosphopeptides, all of which contained phosphotyrosine. The disparity in all these results may reflect the differing phosphorylating conditions among the intact cell, the detergent solubilized cell and the membrane fraction from the cell.

Multiple phosphorylation of the EGF receptor-kinase could provide for functional regulation at each phosphorylation step. Thus the phosphorylation of a given site on the receptor-kinase may alter its interaction with its other phosphorylatable substrates or modulators.

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References

1. Carpenter, G., and Cohen, S. (1979) Ann Rev Biochem 48, 193-216.
2. Carpenter, G., and Cohen, S. (1978) in Biochemical Actions of Hormones (Litwack, G., ed.) Vol. 5, pp. 203-247, Academic Press, New York.
3. King, L.E., and Carpenter, G.F. (1982) in Biochemistry and Physiology of the Skin (L. Goldsmith, ed) Oxford University Press, in press.
4. Carpenter, G., King, L., and Cohen, S. (1978) Nature 276, 409-410.
5. Carpenter, G., King, L., and Cohen, S. (1979) J Biol Chem 254, 4884-4891.

6. King, L.E., Carpenter, G., and Cohen, S. (1980) Biochemistry 19, 1524-1528.
7. Cohen, S., Carpenter, G., and King, L. (1980) J Biol Chem 255, 4834-4842.
8. Cohen, S., Ushiro, H. and Stoscheck, C. (1982) J Biol Chem, in press.
9. Carpenter, G., Poliner, L., and King, L. (1980) Mol Cell Endocrinol 18, 189-199.
10. Fernandez-Pol, J.A. (1981) J Biol Chem 254, 9742-9749.
11. Hunter, T., and Cooper J.A. (1981) Cell 24, 741-752.
12. Gill, G.N., and Lazar, C.S. (1981) Nature 293, 305-307.
13. Linsley, P.S. and Fox, C.F. (1980) J Supramol Struct 14, 461-471.
14. Savage, C.R., and Cohen, S. (1972) J Biol Chem 247, 7609-7611.
15. Bradford, M.M. (1976) Anal Biochem 72, 248-254.
16. Laemmli, U.K. (1970) Nature 227, 680-685.
17. Tuazon, P.T., Merrick, W.C., and Traugh, J.A. (1980) J Biol Chem 255, 10954-10958.
18. Hunter, T. and Sefton, B.M. (1980) Proc Natl Acad Sci USA 77, 1311-1315.
19. Noel, D., Nikaido, K., and Ames, G.F. (1979) Biochemistry 19, 4159-4165.
20. DeJong, W.W., Zweers, A., and Cohen, L.H. (1978) Biochem Biophys Res Commun 82, 532-539.
21. Hofmann, F., Beavo, J.A., Bechtel, P.J., and Krebs, E.G. (1975) J Biol Chem 250, 7795-7801.
22. Rangel-Aldao, R., Kupiec, J.W., and Rosen, O.M. (1979) J Biol Chem 254, 2499-2508.
23. Gressner, A.M., and Wool, I.G. (1974) J Biol Chem 249, 6917-6925.
24. Ushiro, H., and Cohen, S. (1980) J Biol Chem 255, 8363-8365.
25. Reynolds, F.H., Todaro, G.J., Fryling, C., and Stephenson, J.R. (1981) Nature 292, 259-262.