Different Forms of the Epidermal Growth Factor Receptor Kinase Have Different Autophosphorylation Sites†

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Received January 29, 1985

ABSTRACT: Limited proteolysis converts the native (M_r 170000) epidermal growth factor (EGF) receptor to the M_r 150000 form of the receptor. Calcium-activated, neutral protease (purified to homogeneity from beef lung), chymotrypsin, and elastase were all similarly effective in generating the 150-kilodalton (150-kDa) form of the receptor in detergent-solubilized, membrane vesicles shed from A-431 cells. The rate of autophosphorylation with [γ-^{32}P]ATP of the 150-kDa form was only 10% of the rate with the native receptor. This decreased rate was not due to loss of kinase activity, since the phosphorylation of angiotensin was virtually unchanged after limited proteolysis of the native receptor kinase. However, maps of elastase-produced peptides from 170-kDa forms and elastase-generated 150-kDa forms of the EGF receptor showed that the major autophosphorylation sites in these two forms were totally different. Confirming this difference in autophosphorylation sites was the finding that the ^{32}P label in the autophosphorylated native receptor could not be recovered in the 150-kDa form following proteolysis. This label was quantitatively recovered in 30–15-kDa peptide fragments generated simultaneously with the 150-kDa form of the receptor. Therefore, the decreased autophosphorylation of the 150-kDa form results from the loss of preferred autophosphorylation sites on the native receptor. Only 1–3% of the phosphate incorporated in the native receptor during autophosphorylation could be found on the 150-kDa autophosphorylation sites. Hence, autophosphorylation of the tyrosine sites in the 150-kDa form of the EGF receptor is markedly enhanced by removing the major sites autophosphorylated on the native form of the receptor.

The binding of epidermal growth factor (EGF)[1] to its receptor activates a protein kinase (Carpenter et al., 1979) that phosphorylates tyrosine residues (Ushiro & Cohen, 1980) on the receptor itself (Cohen et al., 1980, 1982) as well as on other protein substrates. This protein kinase may mediate many of the cellular effects of EGF, since adding EGF to intact cells increases the amount of phosphotyrosine both on the receptor and on other cellular proteins (Hunter & Cooper, 1981). The EGF-activated protein kinase is an intrinsic part of the receptor (Cohen et al., 1982; Buhrow et al., 1982) and shows striking sequence homology (Downward et al., 1984; Lin et al., 1984; Xu et al., 1984) to the v-*erb*-B oncogene product. v-*erb*-B is a homologous member (Yamomoto, 1983) of the v-*src* class of oncogenes which code for autophosphorylating, tyrosine-specific protein kinases. On the basis of these homologies, the sequence of amino acids around the major autophosphorylation site in pp60$^{v\text{-}src}$ has been used to predict the autophosphorylation site in the EGF receptor. The deduced amino acid sequence of the EGF receptor (Ullrich et al., 1984) suggests several tyrosine residues for autophosphorylation. One of these tyrosines is 342 residues from the carboxyl terminus, and the sequence of amino acids around this tyrosine is homologous with the major site of v-*src* protein kinase autophosphorylation (Smart et al., 1981). However, this site has not been found to be one of the major autophosphorylation sites in the native EGF receptor (Downward et al., 1984).

There are two forms of the EGF receptor whose autophosphorylation is increased when EGF binds to the receptor (King et al., 1980). The smaller of these forms (150 kDa) results from a limited proteolysis (Linsley & Fox, 1980) of the larger native form (170 kDa). While several different proteases can produce the smaller receptor form in vitro (O'Keefe et al., 1981), an endogenous protease that generates the 150-kDa form is activated when cells are broken in the presence of calcium and has been identified as CANP (Gates & King, 1982a; Cassel & Glaser, 1982). Recent work (Chinkers & Brugge, 1984; Basu et al., 1984) has shown that trypsin removes the tyrosine phosphorylation sites on the native EGF receptor while generating the 150-kDa form. These observations agree with the finding (Downward et al., 1984) that the major in vivo or in vitro autophosphorylation sites in the native receptor are within 120 amino acids of the carboxyl terminus. From the above evidence, the major autophosphorylation sites in the native receptor cannot be part of the 150-kDa EGF receptor. Therefore, new major sites for tyrosine autophosphorylation must be present in the 150-kDa form of the receptor to account for its EGF-stimulated autophosphorylation. One of these major new autophosphorylation sites may be the tyrosine residue corresponding to the principal autophosphorylation site in pp60$^{v\text{-}src}$ which was not autophosphorylated in the native EGF receptor.

This report shows that major new sites for tyrosine autophosphorylation are indeed present and utilized in the 150-kDa

† This work was supported by funds from the Veterans Administration (to R.E.G. and L.E.K.) and by U.S. Public Health Service Grant AM-26518 (to L.E.K.).

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[1] Abbreviations: EGF, epidermal growth factor; CANP, calcium-activated neutral protease; ^{125}I-EGF, epidermal growth factor covalently labeled with ^{125}I; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid.

form of the EGF receptor. In the native receptor, we find that only a very small amount of phosphate is incorporated into these cryptic 150-kDa sites during autophosphorylation with ATP. Therefore, the most effective way of increasing the autophosphorylation of these cryptic 150-kDa sites is to generate the 150-kDa form by limited proteolysis of the native receptor.

Materials and Methods

Materials. Mouse EGF, which was provided by Dr. Stanley Cohen, Vanderbilt University, was prepared as described previously (Savage & Cohen, 1972) or was purchased from Collaborative Research. The A-431 human epidermoid carcinoma cells were provided originally by Dr. J. DeLarco, National Institutes of Health. Dulbecco's modified Eagle's medium, calf serum, and penicillin–streptomycin were obtained from Grand Island Biological Co. New England Nuclear supplied the [γ-^{32}P]ATP at a specific activity of 1000–3000 Ci/mmol and ^{125}I as iodide (carrier free). SDS–PAGE protein standards for molecular weight determinations were purchased from Bio-Rad Laboratories. Synthetic leupeptin, bovine pancreas chymotrypsin [N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone treated], porcine pancreas elastase, and Reactive Red-120 agarose were obtained from Sigma Chemical Co. DEAE-cellulose resin was obtained from Whatman, hexylamine- and octylamine–agarose resins were from P-L Biochemicals, and Sephadex G-150 was from Pharmacia Fine Chemicals. TLC (0.1-mm cellulose) plates (20 × 20 cm) were purchased from EM Reagents. All other reagents and chemicals were reagent grade and were purchased from common suppliers.

Purification of Calcium-Activated Neutral Protease. CANP was purified to homogeneity from the supernatant of a beef lung homogenate by a procedure using a sequence of five column chromatography steps (King & Gates, 1985). The first four columns contained DEAE-cellulose, hexylamine–agarose, octylamine–agarose, and Sephadex G-150, respectively, and were loaded and eluted exactly as described by Waxman (1980). The final column of Reactive Red-120 agarose was adapted from Hathaway et al. (1982).

Labeling and Proteolysis of the EGF Receptor. Shed membrane vesicles were prepared from attached A-431 cells by the method of Cohen et al. (1982). ^{125}I-EGF was covalently coupled to the EGF receptor in these vesicles (24 μg of protein) by incubating the vesicles at 30 °C for 30 min at pH 7.4 in a volume of 24–30 μL with ^{125}I-EGF (2.5–10 ng), iodinated as described by Comens et al. (1982). The EGF receptor in A-431-shed membrane vesicles (24 μg of protein) was labeled by autophosphorylation from [γ-^{32}P]ATP (2–20 μCi) at 0 °C in the presence or absence of 36 ng of EGF in buffers containing indicated amounts of $MnCl_2$, Na_3VO_4, Nonidet P-40, and BSA. The phosphorylation reaction was terminated by adding a 50% molar excess of Na_2EDTA (pH 7) over $MnCl_2$ or by adding an equal volume of 2× Laemmli (1970) sample buffer and heating to 100 °C for 2 min. The EGF receptor in A-431-shed membrane vesicles, solubilized in Nonidet P-40, was degraded by using CANP in the presence or absence of calcium or by using chymotrypsin or elastase. Incubation with each protease was at 0 °C, and the reactions were terminated by adding 60 μL of 2× concentrated Laemmli (1970) sample buffer and heating at 100 °C for 2 min. Chymotrypsin and elastase samples were dissolved in water immediately before use while CANP was added in a 1:1 mixture of buffer A (King & Gates, 1985) and glycerol. In CANP experiments when CANP was not added, an appropriate aliquot of a 1:1 mixture of buffer A and glycerol was added.

Analysis and Quantitation of Receptor Labeling. Samples for SDS–PAGE were analyzed according to the method of Laemmli (1970) using 6% or 5–20% gradient gels. Some 6% gels were run, fixed, stained, dried, and autoradiographed as described previously (Gates & King, 1982b). ^{125}I in labeled bands excised from dried gels after autoradiography was quantitated by using a γ spectrometer. Similarly, the radioactivity in ^{32}P-labeled bands excised from gels was determined by using a Nuclear Chicago (Model 470) gas flow detector. With some 6% gels and with all 5–20% gradient gels, autoradiography was done at 4 °C on gels which were not fixed, stained, or dried. The amount of ^{32}P in labeled bands in these wet gels was quantitated as described above. Protein concentration was assayed according to Bradford (1976).

For two-dimensional mapping of phosphorylated peptides, appropriate labeled bands were sliced from 6% gels which were not fixed, stained, or dried. These slices were fixed in 50% CH_3OH and 1% acetic acid and then washed thoroughly in water. Labeled peptides were removed from the gel slices and prepared for peptide mapping as described by Tuazon et al. (1980), except that trypsin was replaced with elastase (total of 20 μg per gel slice). Peptide maps were generated by using electrophoresis and chromatography as described by Hunter & Sefton (1980). A Savant Instruments, Inc. TLE 20 thin-layer electrophoresis apparatus was used for the electrophoresis step which was run at 1000 V and was terminated when bromophenol blue spotted as a dye marker had migrated 8 cm. Following autoradiography of the dried thin-layer plates, appropriate areas of cellulose corresponding to labeled spots were removed and ^{32}P-labeled peptides quantitated by scintillation counting in Ready Solv EP (Beckman Instruments, Inc.). Samples for phosphoamino acid analysis were prepared from gel regions in exactly the same way as the samples for peptide mapping except that trypsin was used instead of elastase. The samples were treated with 6 N HCl at 110 °C and analyzed by electrophoresis at pH 3.5 as described by Cooper et al. (1983).

Tyrosine Kinase Assay. The tyrosine kinase activity of the EGF receptor before and after limited proteolysis was assayed by using angiotensin II as a substrate (Wong & Goldberg, 1983) for phosphorylation with [γ-^{32}P]ATP. A-431-shed membrane vesicles were incubated with 2.5 μg of BSA with or without 30 ng of EGF in 10 μL of a solution which was 120 mM in Hepes, pH 7.4, for 5 min at 0 °C. After the addition of 5 μL of 1% (w/v) Nonidet P-40 and incubation for another 5 min at 0 °C, 5 μL of 30 mM $CaCl_2$ and 5 μL of CANP in buffer A were added. After another 5 min at 0 °C, proteolysis was stopped by adding 5 μL of "stopping mixture" (40 mM EGTA and 20 mM leupeptin, pH 7). As a control, this "stopping mixture" was added with the calcium before the CANP was added. Phosphorylation was initiated by the addition of 10 μL of a solution which was 10 mM in angiotensin II, 100 mM in $MgCl_2$, 100 μM in Na_3VO_4, and 80 mM in Hepes, pH 7.4, followed by the addition of 10 μL of 75 μM [γ-^{32}P]ATP at 4 μCi/10 μL. After 10 min at 0 °C, phosphorylation was terminated by the addition of 150 μL of 4% (w/v) trichloroacetic acid and 10 μL of 20 mg/mL BSA. Following centrifugation, 50-μL aliquots of the supernatant were analyzed as described by Roskoski (1984).

Results

Trypsin has been shown (Chinkers & Brugge, 1984; Basu et al., 1984) to remove a 20–15-kDa peptide at the end of the native EGF receptor which contains nearly all of the tyrosines autophosphorylated in this form of the receptor. The remaining portion of the EGF receptor should be the 150-kDa

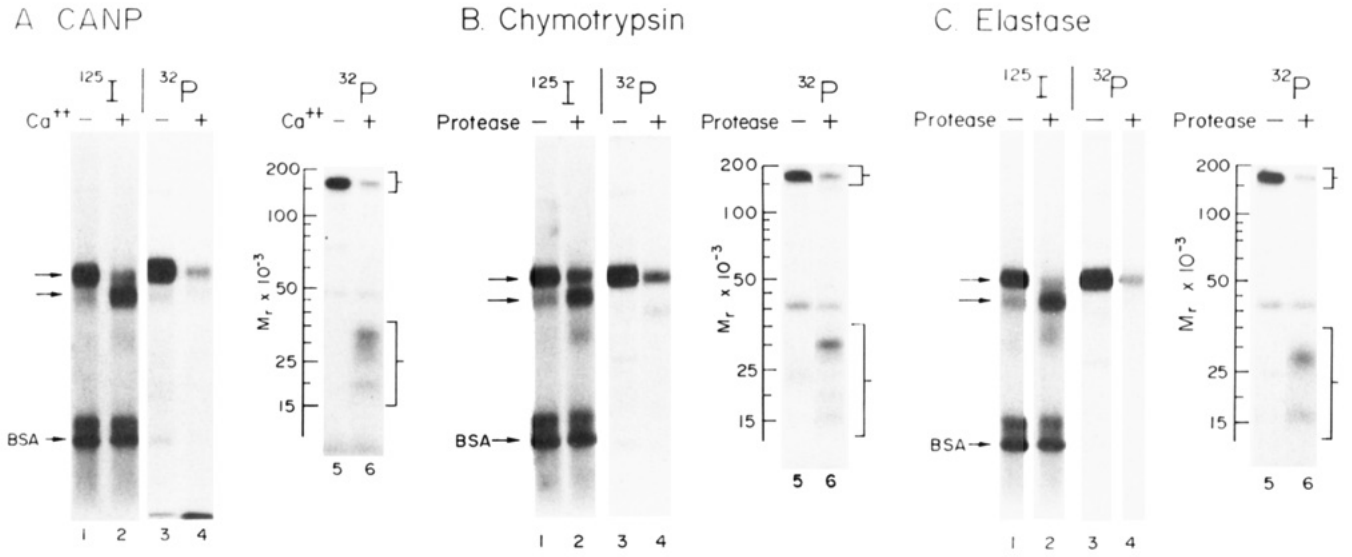

FIGURE 1: Removal of phosphorylated fragments from the autophosphorylating native EGF receptor by limited proteolysis. Autoradiographs of SDS–PAGE gels of vesicles covalently coupled to ^{125}I-EGF (lanes 1 and 2) are compared to similar samples treated with unlabeled EGF and then incubated with [γ-^{32}P]ATP (lanes 3–6). After labeling, every sample in panel A had 6 μL of 0.08 μg/mL CANP added while only samples in lanes 2, 4, and 6 had 6 μL of 35 mM $CaCl_2$ added. In panels B and C, samples in lanes 2, 4, and 6 had 12 μL of 0.05 mg/mL chymotrypsin and 12 μL of 0.04 mg/mL elastase added, respectively. Proteolysis was at 0 °C for 2.5 min in a total volume of 60 μL. Aliquots of the phosphorylated samples were analyzed in lanes 3 and 4 on 6% acrylamide gels and in lanes 5 and 6 on 5–20% gradient acrylamide gels. The square brackets (lanes 5 and 6) indicate the gel regions excised to compare the radioactivity detected before and after protease digestion. The amount of radioactivity appearing in the lower region (lane 6 minus lane 5) divided by the amount of radioactivity removed from the upper region (lane 5 minus lane 6) was used to calculate the recovery of phosphorylated fragments: (A) 12 317 cpm/13 592 cpm = 0.91; (B) 2190 cpm/2008 cpm = 1.09; (C) 3248 cpm/3237 cpm = 1.00. The labeling reactions were as follows. 2.5 ng of ^{125}I-EGF was incubated with vesicles in 28.5 μL of a solution containing 6 μL of 200 mM Hepes, pH 7.4, 10 mM $MnCl_2$, and 200 μM Na_3VO_4 along with 6 μL of a solution which was 1% Nonidet P-40 and 50% glycerol. The covalent coupling was stopped by the addition of 33 ng of unlabeled EGF and after 5 min cooling to 0 °C. The samples for autophosphorylation were treated identically except that unlabeled EGF replaced ^{125}I-EGF. Autophosphorylation followed using 0.04 μM [γ-^{32}P]ATP (4–5 μCi) for 2.5 min in a volume of 42 μL and was stopped by the addition of 6 μL of 15 mM EDTA, pH 7. The samples labeled with ^{125}I-EGF were treated identically except water replaced the [γ-^{32}P]ATP.

form which can also be autophosphorylated (King et al., 1980; Cohen et al., 1980), presumably on tyrosine residues not located on the 20–15-kDa peptide which was removed from the native receptor. To study the 150-kDa autophosphorylation sites, we used three proteases which, in contrast to trypsin, do not appear to rapidly degrade the EGF receptor to forms even smaller than 150 kDa. One of these proteases was CANP, the endogenous protease known to degrade EGF receptors when cells are broken in the presence of calcium (Gates & King, 1982a; Cassel & Glaser, 1982). CANP was purified to homogeneity (King & Gates, 1985) from beef lung and showed only two protein bands on SDS–PAGE which corresponded to the 80- and 30-kDa subunits of the heterodimer previously reported for this protease (Waxman, 1980; Hathaway et al., 1982). Also in agreement with other reports, the purified CANP required millimolar calcium levels for activation and was inhibited by leupeptin and iodoacetic acid. Based on the amount of protein, this purified protease was as active as either chymotrypsin or elastase during degradation of the native EGF receptor (see Figure 1).

To establish the fate of autophosphorylation sites on the native receptor during controlled proteolysis, we labeled the EGF binding sites and the autophosphorylation sites of the receptor separately. The native receptors in identical aliquots of A-431-shed membrane vesicles were labeled either at the EGF binding site by using the covalent coupling of ^{125}I-EGF or at the tyrosine autophosphorylation site by using [γ-^{32}P]-ATP. The labeled membrane vesicles were then treated with CANP, chymotrypsin, or elastase for 2.5 min at 0 °C and were analyzed by SDS–PAGE as shown in Figure 1. In the absence of calcium, CANP had no effect on the amount of 170-kDa receptor whether labeled with ^{32}P or ^{125}I-EGF (data not shown). In the presence of calcium, CANP converted 72% of the original 170-kDa receptor covalently coupled to ^{125}I-EGF to the 150-kDa form while converting the ^{32}P label in the autophosphorylated 170-kDa receptor quantitatively to ^{32}P-labeled peptides of 30–15 kDa (Figure 1A). Nearly identical results were obtained with a separate preparation of purified CANP. Similar results were obtained with chymotrypsin (Figure 1B) and with elastase (Figure 1C) except the conversion of the 170–kDa EGF receptor labeled with ^{125}I-EGF to the 150-kDa form was reduced to 58% with either protease. Again, the ^{32}P label in the autophosphorylated receptor was quantitatively converted to ^{32}P-labeled peptides and was not found with the 150-kDa form of the receptor. Increasing or decreasing the concentrations of the three proteases by 50% produced less 150-kDa receptor covalently coupled to ^{125}I-EGF. Apparently there is an optimum protease concentration which maximizes the production of the 150-kDa form while minimizing its subsequent degradation. Even when the protease concentration was varied, ^{32}P was never found with the 150-kDa receptor. While producing the 150-kDa form of the EGF receptor, these proteases remove a small peptide(s) which has (have) virtually all the tyrosine sites autophosphorylated in the native 170-kDa receptor.

The sum of the molecular weights for 150-kDa receptor and the 30-kDa fragments does not equal the molecular weight of the native receptor. We have shown (Gates & King, 1982b) that the apparent molecular weight of the native receptor increases as much as ,12 kDa as a result of autophosphorylation. As seen above, all of the phosphate incorporated during autophosphorylation of the native receptor is on the 30–15-kDa fragments. It is likely that the phosphate on these fragments increases their molecular weight even more dramatically and thereby explains the discrepancy in the summed molecular weights.

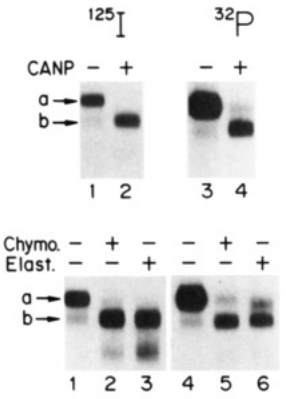

FIGURE 2: Autophosphorylation of the protease-generated 150-kDa receptor. Selected regions of autoradiographs of SDS–PAGE gels on which vesicles covalently coupled to ^{125}I-EGF (lanes 1 and 2) and treated with proteases are compared to similar samples treated with unlabeled EGF followed by proteases and then incubated with [γ-^{32}P]ATP (lanes 3 and 4). In the upper panel, every sample had 6 μL of 30 mM $CaCl_2$ added while only the samples in lanes 2 and 4 had 9 μL of 0.2 mg/mL CANP added. In the lower panel, samples in lanes 2 and 5 had 9 μL of 0.04 mg/mL chymotrypsin added while samples in lanes 3 and 6 had 9 μL of 0.02 mg/mL elastase added. The arrows designated "a" and "b" point to the 170- and 150-kDa forms of the EGF receptor, respectively. The labeling reactions were as follows: 4.8 ng of ^{125}I-EGF was incubated with 24 μg of vesicles in 24 μL of 50 mM Hepes, pH 7.4, for 30 min at 30 °C. After being cooled to 0 °C, 6 μL of 10 mM $MnCl_2$ and 200 μM Na_3VO_4 was added. Following proteolysis for 5 min at 0 °C in a volume of 51 μL, autophosphorylation was done at 0 °C for 30 s by adding 9 μL of 3.75 μM [γ-^{32}P]ATP (2.7 μCi). Proteolysis and phosphorylation were then stopped by the addition of 2× Laemmli (1970) sample buffer and heating to 100 °C. The samples labeled with ^{125}I-EGF were treated identically except water replaced the [γ-^{32}P]ATP.

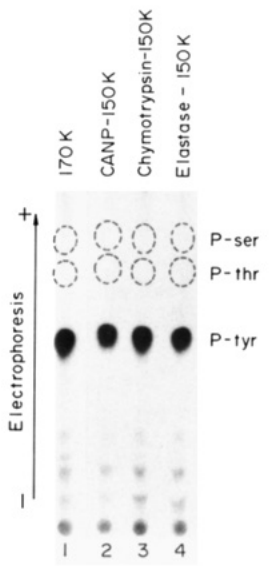

FIGURE 3: Analysis of phosphorylated amino acids in the autophosphorylated 170- and 150-kDa receptor forms. An autoradiograph of the TLC plate used to separate the phosphoamino acids in the native receptor (lane 1) and in the 150-kDa receptor generated by CANP, chymotrypsin, or elastase (lanes 2, 3, and 4, respectively) is shown. The proteolysis and phosphorylation were done as in the legend to Figure 2 except 6 μL of 0.1 mg/mL chymotrypsin or 0.05 mg/mL elastase was added, while 6 μL of 0.2 mg/mL CANP was added for proteolysis and 6 μL of [γ-^{32}P]ATP at 0.3 μM (3.6 μCi) in lane 1 or at 1.5 μM (18 μCi) in lanes 2–4 was added for autophosphorylation.

Table I: Comparison of EGF-Stimulated Autophosphorylation in the 170- and 150-kDa Receptor Forms

	170 kDa[a] untreated control	150 kDa[a] CANP generated	150 kDa[a] chymotrypsin generated	150 kDa[a] elastase generated
% of ^{32}P labeling compared to the 170-kDa form[b]	100	11.6 ± 8.7	9.4 ± 2.2	9.5 ± 2.3
n[d]	7	4	3	3
x-fold increase in ^{32}P labeling due to EGF[c]	19.9 ± 6.1	13.0 ± 6.3	22.6 ± 5.7	19.5 ± 9.9

[a] ^{125}I and ^{32}P labeling in these molecular weight regions from the SDS–PAGE gels shown in Figure 2 (and from other similar experiments) was quantitated as described under Materials and Methods. [b] Mean ± standard deviation. Values given are $A/B \times 100$. A = ^{32}P-labeled sample treated with protease minus untreated sample divided by ^{125}I-labeled sample treated with protease minus untreated sample for the 150-kDa region of gel lanes. B is the same computation for the 170-kDa region of gel lanes. [c] Mean ± standard deviation. Values are the x-fold increase due to EGF calculated from the amount of ^{32}P labeling in the indicated molecular weight regions for samples autophosphorylated in the presence of EGF divided by the corresponding amount for identical samples autophosphorylated in the absence of EGF. [d] n = number of experiments.

Even though the 150-kDa form of the EGF receptor has lost all the tyrosine sites autophosphorylated in the native 170-kDa receptor, it still autophosphorylates itself whether generated by CANP, chymotrypsin, or elastase (King & Gates, 1985). Figure 2 also shows these data which suggests that the autophosphorylation of the 150-kDa receptor form is reduced compared to the 170-kDa native receptor. Even though the 30–15-kDa fragments were present in the reaction mixture after proteolysis, no incorporation of ^{32}P into these fragments was detected (data not shown). As with the native receptor, this autophosphorylation was only on tyrosine residues (Figure 3) and was stimulated over 10-fold by the presence of EGF (Table I). Apparently the 150-kDa EGF receptor form can be autophosphorylated by the intrinsic receptor kinase only after the tyrosine sites for autophosphorylation in the native receptor are removed.

To quantitate the relative ability of the 170- and 150-kDa receptor forms to autophosphorylate, the native EGF receptor was covalently coupled to ^{125}I-EGF and treated with the proteases. At the same time, unlabeled, native receptor was first converted to the 150-kDa form by incubation with CANP, chymotrypsin, or elastase and then incubated with [γ-^{32}P]ATP in the presence of EGF for 30 s. The relative ability of the two receptor forms to autophosphorylate was calculated from the ratio of ^{32}P to ^{125}I label in a given receptor form following SDS–PAGE (Figure 2). As seen in Table I, the protease-generated 150-kDa form autophosphorylates only one-tenth as well as the native receptor. Within each comparison in this table, the A-431 vesicles, reagents, and conditions were identical except that protease was added to one sample (150-kDa receptor) and not to the other (170-kDa receptor). Multiple comparisons were made by using different batches of A-431 vesicles and of CANP. The reasons for the large variability in the CANP comparisons are not known. Even though the range of CANP-generated 150-kDa autophosphorylation was from 4% to 23%, no correlation of the values was found with either the batch of vesicles or the preparation of CANP. Depletion of [γ-^{32}P]ATP was not responsible for the variability, since increasing the ATP concentration from 0.5 to 2.5 μM decreased the ratio of 150- to 170-kDa autophosphorylation from 23% to 16% in one case and from 6.2% to 4.5% in another case. In spite of the variability in these comparisons, it is clear that the 150-kDa EGF receptor autophosphorylates much less well than the native receptor does.

Having fewer and perhaps less preferred sites for autophosphorylation would obviously explain the decreased rate of autophosphorylation of the 150-kDa EGF receptor. The

Table II: Tyrosine Kinase Activity[a] of 170- and 150-kDa EGF Receptor Forms

	PO_4 incorporated (pmol/assay)	
	170-kDa receptor, CANP inhibited[b]	150-kDa receptor, CANP active[b]
−EGF	2.4[c]	2.1
+EGF	7.9	8.5

[a] Phosphorylation of angiotensin II using [γ-^{32}P]ATP in a volume of 50 μL was determined as described under Materials and Methods. [b] Identical experiments were also analyzed by SDS–PAGE, and ^{32}P labeling of the 170-kDa receptor was quantitated. Compared to labeling in the absence of CANP, labeling after treatment with inhibited CANP was 100.3% or 97.4% for the two different CANP preparations used, and labeling after treatment with active CANP was 1.0% or 0.2% for the two CANP preparations used. [c] Average of two experiments using different preparations of CANP at 1.0 and 1.25 μg per 50-μL assay. For each experiment, the phosphate incorporated was determined from the radioactively labeled angiotensin in two separate aliquots from each of two separate assay tubes. In the absence of angiotensin, the phosphate incorporated was 1.6 and 1.7 pmol in the absence or presence of EGF, respectively. With angiotensin present and CANP inhibited, doubling the amount of vesicles (from 19 to 38 μg of protein) per assay increased phosphate incorporation to 3.2 and 15.0 pmol in the absence or presence of EGF, respectively.

data in Figure 1 show that the native EGF receptor does have preferred autophosphorylation sites which are removed when the 150-kDa receptor is produced. Several other possibilities could explain the decreased rate of autophosphorylation of the 150-kDa EGF receptor. Removal of the 30–15-kDa fragment from the native EGF receptor could induce a conformational change in the remaining portion of the receptor which either reduces the stimulation of the kinase by EGF or makes the kinase less active. The results in Table I show that autophosphorylation is stimulated by EGF to the same extent for the 150-kDa receptor as for the native receptor. Apparently, limited proteolysis of the receptor kinase does not affect EGF stimulation of the kinase. Similarly, the data in Table II indicate that phosphorylation of the exogenous substrate, angiotensin II, is the same for the 150- and 170-kDa receptor forms. Thus, the activity of the kinase does not appear to be decreased by limited proteolysis. Therefore, it is likely that the reduced autophosphorylation of the 150-kDa receptor kinase compared to the native form is simply due to the loss of preferred tyrosine residues for autophosphorylation.

Does the native EGF receptor kinase incorporate any phosphate at all into its cryptic 150-kDa receptor autophosphorylation sites? No phosphate was incorporated into these cryptic sites in the experiments shown in Figure 1 when the native receptor was autophosphorylated with [γ-^{32}P]ATP before limited proteolysis. However, peptide mapping of ^{32}P-labeled sites in the 170- and 150-kDa receptor forms provides a more sensitive approach to answering this question. Peptide mapping can be used to determine if any of the minor ^{32}P-labeled peptides in the 170-kDa form correspond to the major ^{32}P-labeled peptides in the 150-kDa form. The results of such an experiment are shown in Figure 4. Clearly, the major ^{32}P-labeled peptides are totally different when the 170- and 150-kDa receptor forms are autophosphorylated using [γ-^{32}P]ATP. The two arrows in the 170-kDa map (Figure 4A) point to two ^{32}P-labeled peptides which are present in both the 170- and 150-kDa forms after autophosphorylation. In the 170-kDa form, these two labeled peptides represent a very minor fraction of the ^{32}P labeling. In two separate experiments, these ^{32}P-labeled peptides represented only 0.8% and 1.2% of the total ^{32}P label incorporated in the native 170-kDa receptors (Table III). These same two peptides are the major

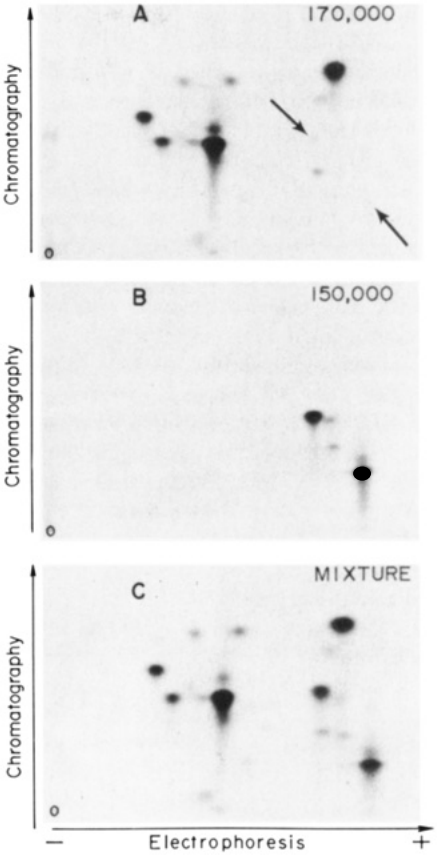

FIGURE 4: Comparison of autophosphorylated sites in the 170- and 150-kDa forms of the EGF receptor. Autoradiographs of the TLC plates used for two-dimensional peptide mapping of elastase-produced peptides are shown. Panel A is the map of the native receptor; panel B is the map of the elastase-generated 150-kDa receptor; panel C is the map resulting from a mixture of the peptides analyzed in (A) and (B). The conditions for proteolysis and labeling were as described in the legend to Figure 3 except 12 μL of [γ-^{32}P]ATP (4.8 μCi) at 0.2 μM was added for labeling of the 170-kDa receptor while 12 μL of [γ-^{32}P]ATP (17 μCi) at 0.7 μM was added for labeling of the 150-kDa receptor.

Table III: Fraction of ^{32}P Incorporated into the 170-kDa Receptor by Autophosphorylation Found on 150-kDa Receptor Sites

expt[a]	[ATP] (μM)	time[b] (min)	^{32}P in 150-kDa sites[c]/ ^{32}P in all sites
1	0.04	0.5	0.008
2	0.04	0.5	0.012
3	0.02	0.5	0.012
	3.1	2	0.029
4	0.02	0.5	0.016
	3.1	2	0.035

[a] Experiments 1 and 2 were done with different batches of A-431-shed membrane vesicles as were experiments 3 and 4. [b] Time of incubation of A-431-shed membrane vesicles (pretreated with EGF and detergent) with [γ-^{32}P]ATP at 0 °C. [c] ^{32}P-labeled peptides on the TLC plates were quantitated as described under Materials and Methods. The radioactivity determined for any area of the TLC plate was always corrected by subtracting the background radioactivity determined for a comparable unlabeled area of the TLC plate.

labeled peptides in the peptide maps of autophosphorylated 150-kDa receptor (Figure 4B). Hence, only a very small amount of phosphate is incorporated into the native receptor's cryptic autophosphorylation sites which become the major autophosphorylation sites on the 150-kDa form.

It is important to note that the peptide maps shown in Figure 4 were produced by using the same protease to first generate the 150-kDa receptor and then to further degrade both the 170- and 150-kDa forms to peptides. The only protease used

to degrade either the 170- or the 150-kDa EGF receptor form in this set of experiments was elastase, so that all the cleavage sites must be specific for elastase. Every peptide produced from the 150-kDa form will be identical with a comparable peptide produced from the 170-kDa form. Therefore, identical autophosphorylation sites in the 170- and 150-kDa forms will be detected as identical ^{32}P-peptides which comigrate during peptide mapping. This method of using the same protease to generate the 150-kDa receptor form and to generate the peptides to be mapped allowed us to locate and quantitate the labeling of the 150-kDa autophosphorylation sites in the 170-kDa receptor form.

Relative autophosphorylation of the minor ^{32}P-labeled peptides in the native EGF receptor was increased by increasing the ATP concentration from 0.02 to 3.1 μM and the labeling time from 30 s to 2 min. Autophosphorylation of the 150-kDa sites in the 170-kDa receptor was evaluated from peptide maps similar to those shown in Figure 4. At the higher ATP level, the total phosphate incorporated into the 170-kDa receptor increased more than 10-fold while the phosphate incorporated into the cryptic 150-kDa sites increased 20–25-fold. Therefore, the higher ATP level and longer labeling time increased the amount of phosphate incorporated into the cryptic 150-kDa sites relative to all the sites on the 170-kDa receptor from 1.2% to 3.2% (Table III). Compared to the lower level of ATP, the higher level increased the apparent molecular weight determined by SDS-PAGE of nearly all of the ^{32}P-labeled 170-kDa receptor but did not affect the corresponding 150-kDa receptor (data not shown). This increase in apparent molecular weight as determined by SDS-PAGE is caused by phosphorylation of multiple tyrosines in a single 170-kDa receptor molecule (Gates & King, 1982b). Therefore, the higher ATP level was sufficient to multiply phosphorylate the native receptor. Even though more than one of the preferred sites was phosphorylated per molecule during autophosphorylation of the native EGF receptor, only 3% of the phosphate incorporated was found on the cryptic 150-kDa sites.

Discussion

This report shows that limited proteolysis of the native EGF receptor (170-kDa) results in a form of the receptor (150 kDa) whose major autophosphorylation sites are totally different from the major autophosphorylation sites on the native receptor. Peptide mapping of the in vitro autophosphorylation sites showed that the major sites in the 150-kDa form were only very minor sites in the 170-kDa form. The reason for these differences is that limited proteolysis using CANP, chymotrypsin, or elastase removed from the native receptor a small fragment that contains nearly all of the sites autophosphorylated in the native receptor. Others (Chinkers & Brugge, 1984; Basu et al., 1984) have reported that trypsin also removes a small fragment from the native receptor that contains the major autophosphorylation sites. Even though the major autophosphorylation sites of the native receptor were removed, the 150-kDa form of the receptor generated by the proteases used in this report still phosphorylated itself on tyrosine residues. The rate of this EGF-stimulated autophosphorylation was only about 10% of the autophosphorylation rate of the native receptor. Cohen et al. (1982) estimated that the autophosphorylation rate of the 150-kDa receptor generated during cell lysis in the presence of calcium was only 10–20% of the rate for the native receptor isolated in shed membrane vesicles. We did not find either an increased or a decreased rate of phosphorylation of angiotensin II by the 150-kDa form compared to the rate with the native form of the EGF receptor. Therefore, the reduced autophosphorylation rate in the 150-kDa receptor is not the result of decreased tyrosine kinase activity. While this reduced autophosphorylation rate might be explained by the simple numerical loss of preferred autophosphorylation sites, another possibility is that the 150-kDa receptor autophosphorylates by an intermolecular mechanism in contrast to the intramolecular autophosphorylation of the native receptor (Weber et al., 1984).

The only support for the hypothesis that autophosphorylation of the 150-kDa receptor form occurs in vivo is derived from the EGF-stimulated autophosphorylation that occurs in vitro. However, a one to one correspondence of in vivo and in vitro tyrosine phosphorylation sites has not been observed in the autophosphorylation of either EGF receptor kinase (Downward et al., 1984) or v-*src* kinase (Graziani et al., 1983). In these cases, minor sites of autophosphorylation in vivo were often found as major autophosphorylation sites in vitro. Graziani et al. (1983) suggested that these discrepancies between the in vivo and in vitro sites of autophosphorylation can be resolved by using [γ-^{32}P]ATP levels of 1 μM or less for in vitro labeling. The autophosphorylation of the 150-kDa site on the native receptor may be just such an in vitro artifact since decreasing the ATP level decreases its autophosphorylation relative to the total amount of phosphate incorporated. On the other hand, decreasing the ATP level increases the autophosphorylation of the same site in the 150-kDa form of the receptor relative to the amount of phosphate incorporated into the 170-kDa receptor. Therefore, autophosphorylation of the 150-kDa site in the native receptor may be an in vitro artifact resulting from the use of high ATP levels, while autophosphorylation of the same site in the 150-kDa form of the receptor is not such an artifact.

Neither the 150-kDa form of the EGF receptor nor the autophosphorylation of this form has been detected in the intact cell, even though autophosphorylation (Hunter & Cooper, 1981) and degradative loss (Stoscheck & Carpenter, 1984) of the native receptor are readily detected. Of course, tyrosine phosphate on the 150-kDa receptor may be difficult to detect in vivo, since this receptor form retains virtually none of the tyrosine phosphate from the native receptor and autophosphorylates only one-tenth as well as the native receptor. Detection of the autophosphorylated 150-kDa receptor is also hindered by its further degradation in vivo to smaller receptor forms and fragments. Because it is both generated and eliminated by receptor degradation, the 150-kDa receptor form is likely to be, at any one time, only a small fraction of the total amount of native and degraded receptor forms. This is apparently the case in vivo, since no smaller EGF receptor forms were detected during degradation of the metabolically labeled native receptor (Stoscheck & Carpenter, 1984). The transient and elusive nature of an autophosphorylated 150-kDa form of the EGF receptor would not exclude it as an intermediate essential to the mechanism of EGF action.

A tentative role for autophosphorylation of the 150-kDa receptor has been suggested by the results of Basu et al. (1984). They found that limited proteolysis of the EGF receptor with trypsin generated a 42-kDa fragment that did not retain the EGF binding site or the major autophosphorylation sites. However, this fragment retained kinase activity and the ability to autophosphorylate itself. At present, a biological role for this tyrosine kinase fragment derived from the EGF receptor is speculative since this kinase was not stimulated by EGF and retained only 28% of the activity present in the native receptor. An attractive hypothesis is that, like v-*src* kinase (Purchio et

al., 1983), extensive autophosphorylation of tyrosine sites in the kinase fragment will activate this kinase. If, as appears likely, the sites of autophosphorylation on this tyrosine kinase fragment are identical with the 150-kDa autophosphorylation sites, then they will be more readily phosphorylated in the 150-kDa receptor form than in the 170-kDa form (compare results in Tables I and III). In contrast to the kinase in the 42-kDa fragment, the kinase in the 150-kDa receptor form is stimulated by EGF and has the same activity as in the native receptor (see Tables I and II). Therefore, autophosphorylation of the tyrosine sites in the 42-kDa kinase fragment will occur most readily in the 150-kDa receptor form before the fragment is proteolytically removed.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Rebecca Gariglietti and Linda Coats. We thank Dr. Stanley Cohen for many helpful discussions.

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