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ALKALINE HYDROLYSIS AND MULTIPLE SITE AUTOPHOSPHORYLATION DIFFER FOR TWO FORMS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

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Summary: Different tyrosines are autophosphorylated on the native and on the protease-generated 150 kDa forms of the epidermal growth factor receptor. High ATP concentrations increase the apparent molecular weight of already phosphorylated native receptors but not of the 150kDa form, indicating that only the native receptor has multiple autophosphorylation sites available. The non-identity of the tyrosine-phosphates on the native and 150kDa receptor forms is seen in their response to alkaline hydrolysis (10% and 40% resistant, respectively). Since the liberated phosphate is peptide bound, the native receptor fails to be alkali-resistant because of which peptide bonds are hydrolyzed.

The binding of EGF to its specific receptor (reviewed in ref. 1) activates (2,3) the tyrosine-specific protein kinase (4) portion of the receptor (5,6) on the inside of the cell (7). This activation increases the phosphorylation of a limited number of cellular proteins (8) as well as of the receptor itself (5,8). The native receptor is autophosphorylated on three tyrosine residues near the carboxyl terminal receptor end (9). These tyrosine residues are on (10,11) the 20kDa fragment removed from the cytoplasmic end of the native receptor during limited proteolysis (7,12). The remaining 150kDa portion of the receptor phosphorylates itself on a new tyrosine residue which is not phosphorylated to a significant degree in the native receptor (13).

Little is known about the autophosphorylation site in the 150kDa form of the EGF receptor except that it is on a tyrosine residue which is not present in the protease-generated 130kDa form of the receptor (13). This report describes differences we observed between the two autophosphorylating forms of the EGF receptor following their separation by SDS-PAGE.

Abbreviations Used: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Methods

The A-431 human epidermoid carcinoma cells were obtained initially from Dr. J. DeLarco, National Institutes of Health. Mouse EGF was purchased from Collaborative Research while [γ-^{32}P]ATP at a specific activity of 1000-3000 Ci/mmole and carrier-free $^{32}PO_4$ as orthophosphoric acid were from New England Nuclear. Acrylamide, SDS and the molecular weight standards for PAGE were provided by Bio-Rad Laboratories. Pharmacia Fine Chemicals supplied Sephadex G-50 and blue dextran 2000. Horse heart cytochrome C and muscle myoglobin and bovine erythrocyte carbonic anhydrase were supplied by Sigma Chemical Company while bovine serum albumin (BSA) came from ICN Immunobiologicals. BDH Chemicals LTD. provided Nonidet P40.

As previously reported (14,15), A-431 cells were cultured and grown to confluency and then plasma membranes were prepared according to Thom et al (16). An amount of membranes equivalent to 48μg of protein were phosphorylated at 0°C in the presence of 20mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid at pH=7.4, 1mM $MnCl_2$, 20μM Na_3VO_4, and 50μg/ml BSA. When present EGF at a final concentration of 0.6μg/ml was always added before the Nonidet P-40. The phosphorylation reaction was initiated by the addition of 6 to 24μCi of [γ- ^{32}P]ATP along with an unlabeled amount of ATP sufficient to give a final concentration ranging from 0.01 to 5μM in a total reaction volume of 120μl. After proceeding for 0.5 to 2.5 min the reaction was terminated by the addition of 120μl of 2x Laemmli sample buffer (17) followed by immediate heating in a 100°C water bath for 2 min. As previously described (13-15), this assay mixture was separated by SDS-PAGE and the gels were dried and subjected to autoradiography.

To establish which phosphorylated bands were alkali-resistant, gels were incubated at 55°C for 2 hrs in 0.5N KOH after staining and destaining but before drying and autoradiography. To collect the hydrolyzed material removed by treating with alkali, phosphorylated bands detected in gels which were autoradiographed before fixation and drying were excised. The labeled proteins were then fixed in the gel slice with a solution of 50% methanol, 1% acetic acid and alkali-treated as above. After this treatment the gel slices were washed 3x with water and once with 0.1N H_3PO_4 overnight. The alkali extract and the combined washes were adjusted to pH=11, lyophilized and reconstituted with 1 ml of water. The reconstituted extract was analyzed on a 40 ml column (1x48 cm) of Sephadex G-50 by eluting with 0.1M potassium phosphate buffer at pH=11 while collecting 1 ml fractions. The radioactivity in these gel slices, in the combined washes and column fractions as well as in appropriate regions of individual gel lanes excised from dried gels were all determined by counting Cerenkov radiation.

Results and Discussion

During the proteolytic conversion of the native receptor to the 150kDa form, we (13) found that the three autophosphorylation sites (9) of the native receptor were removed. While new tyrosines must be autophosphorylated in the smaller receptor form, neither their location nor number is known. Since we had shown (18) that the native receptor increases its molecular weight by as much as 12kDa on SDS-PAGE because multiple sites are autophosphorylated, a similar approach to determining if the smaller receptor form had multiple autophosphorylation sites was tried.

As shown in Fig. 1A (lanes 1 & 3) autophosphorylation at high rather than low ATP concentrations increased the apparent molecular weight of the upper labeled band which corresponds to the native receptor. Since the EGF receptor was detected on the gel by its labeling with radioactive phosphate, the apparent molecular weight of an already labeled and therefore already phosphorylated receptor increased. It follows that there must be multiple phosphorylation sites on the native receptor. This interpretation agrees with the 3 autophosphorylated tyrosines found in the native receptor (9) and with the well-known increase in molecular weight determined by SDS-PAGE for several proteins following phosphorylation.

There was no increase in apparent molecular weight when the 150kDa form of the receptor was autophosphorylated at high versus low ATP concentrations (compare lower band in lanes 1 & 3, Fig. 1A). Furthermore, high levels of unlabeled ATP added to both

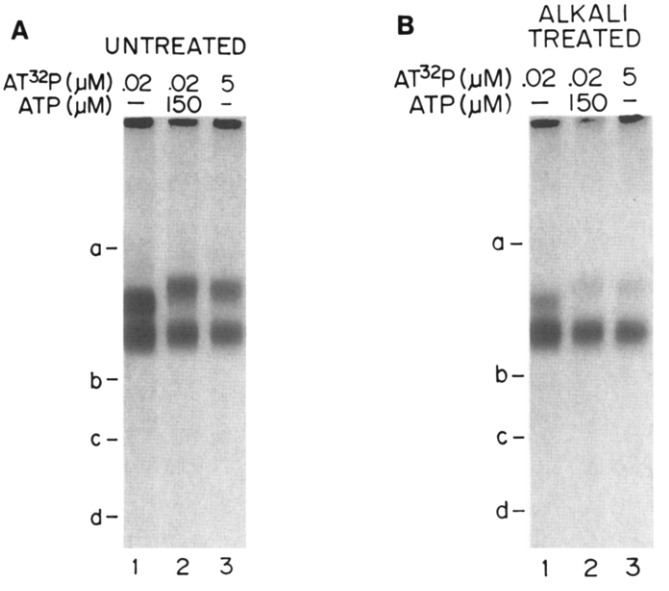

Figure 1: Effect of alkaline treatment of gels on the labeled EGF receptor forms.

A-431 membranes were labeled in the presence of EGF and 0.1% Nonidet P-40 and analyzed on gels as described in **Methods** with 100μl aliquots from the same reaction tube being analyzed in the identically numbered lanes in panel A and B. Only the gel in panel B was alkali-treated. Autoradiographs of the dried gels which were developed after 57 hrs (panel A) or 168 hrs (panel B) are shown. The sample in lane 1 was autophosphorylated for 30 sec using 0.02μM [γ-^{32}P]ATP (6μCi). The sample in lane 2 was autophosphorylated identically except that after labeling for 30 sec unlabeled ATP at 150μM final concentration was added for 2 min. The sample in lane 3 was autophosphorylated using 5μM [γ-^{32}P]ATP (24μCi) for 30 sec. a,b,c,d indicate the position on the gel of the molecular weight markers myosin (200kDa), β-galactosidase (116kDa), phosphorylase B (92.5kDa), and BSA (66kDa) respectively.

forms of the receptor which were first autophosphorylated together with low levels of radioactive ATP increased the apparent molecular weight of only the native 170kDa form of the receptor and not of the 150kDa form (lanes 1 & 2 in Fig. 1A). Hence the previously labeled and, therefore, already phosphorylated 150kDa receptor form does not increase its molecular weight when exposed to high levels of ATP. Either phosphorylation of additional tyrosines does not increase its molecular weight or only one tyrosine is autophosphorylated in the 150kDa receptor form.

There are two potential autophosphorylation sites in the 150kDa receptor form. These sites were identified using the known sequence of the native EGF receptor (19) and assuming acidic amino acids must be at the first and fourth positions on the amino terminal side of the tyrosine (20). One of these sites is tyrosine 992. This tyrosine may be removed along with the 3 tyrosines known (9) to be autophosphorylated in the native receptor by the proteases which generate the 150 kDa receptor form since the exact sites of cleavage by the proteases are not known. Tyrosine 845 is the other potential site. It is almost certainly retained in the 150kDa receptor form and may be the only tyrosine autophosphorylated in that receptor form.

Subjecting SDS-PAGE gels containing protein bands labeled with radioactive phosphate to alkaline hydrolysis is a rapid screening method (21) to determine if the incorporated phosphate is on serine (hydrolyzed) or on tyrosine or threonine (neither hydrolyzed). Fig. 1 compares autoradiographs from identical gels of the autophosphorylated forms of the EGF receptor where one gel was alkali-treated (panel B) and one was not (panel A). If the two autophosphorylating forms of the EGF receptor have identical resistance to alkaline hydrolysis, the ratio of radioactivity of the upper to lower band in panel B should be identical to the ratio in panel A. Clearly these ratios are not equal. The diminished upper band in panel B shows that the autophosphorylated 170kDa form of the EGF receptor is apparently more susceptible to alkaline hydrolysis than the autophosphorylated 150kDa form. Furthermore, this decreased resistance to alkaline hydrolysis is observed whether the 170kDa receptor is singly (lane 1) or multiply (lanes 2,3) phosphorylated. Table I presents a quantitative analysis of results similar to those shown in Fig. 1. Independent of the degree of phosphorylation nearly 40% of the tyrosine-PO_4 on the autophosphorylated 150kDa

Table I - Alkali Resistance of the Autophosphorylated EGF Receptor-Kinase Forms

PHOSPHORYLATION CONDITIONS		PERCENT OF PHOSPHATE NOT HYDROLYZED	
[γ-^{32}P] ATP	Unlabeled ATP*	170 kDa Receptor	150 kDa Receptor
0.02 μM	—	11.2%	37.9%
0.02 μM	150 μM	10.0%	38.9%
5.0 μM	—	11.5%	38.5%

*Added 30 sec. after the [γ-^{32}P] ATP

form of the EGF receptor is resistant to alkali hydrolysis while slightly more than 10% is resistant in the 170kDa form.

To determine if the differences in alkali-lability were due to actual hydrolysis of tyrosine-PO_4 the hydrolysate was analyzed for free phosphate. The labeled forms of the receptor were excised from SDS-PAGE gels of three separate membrane preparations and were individually hydrolyzed and the hydrolysates prepared for molecular sieving chromatography as described in the **Methods** section. The average percentages of the total radioactivity remaining in the gel slices after hydrolysis and washing were 11% and 32% for the 170kDa and 150kDa forms respectively while the corresponding percentages in the hydrolysates immediately before column chromatography were 89% and 67%. Desalting and neutralizing the hydrolysate always produced low recoveries of label (data not shown) which precluded analysis of the labeled material by thin layer electrophoresis or chromatography. Hence, analysis was by column chromatography at high pH and high salt concentrations. The results are shown in Fig. 2 for the hydrolysates from one set of labeled 170kDa (-O-) and 150kDa (-●-) forms. Very little of the radioactivity is recovered in the column void volume or as phosphate. It follows that phosphate is not being hydrolyzed from tyrosine nor is the intact receptor being eluted from the gel during hydrolysis.

The failure to detect a protein phosphorylated on tyrosine by the alkaline hydrolysis screening method has been presumed to be due to actual hydrolysis of the tyrosine phosphate because of its local environment (21). Since very little labeled inorganic phosphate is detected in the hydrolysate of the labeled native receptor, this explanation cannot account for the likely failure of this method, as shown in Fig. 1B, to identify the native EGF receptor as being phosphorylated on tyrosine. Since almost all

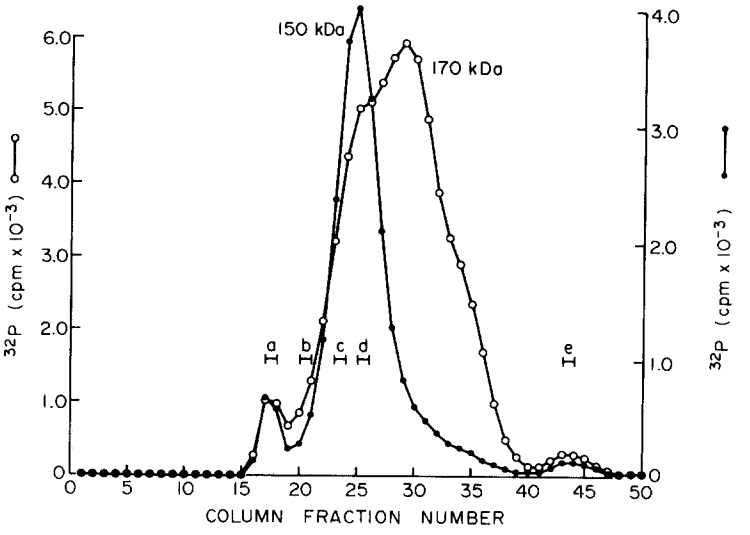

Figure 2: Molecular sieving analysis of the alkaline hydrolysate of gel slices containing the labeled EGF receptor forms.
A-431 membranes were labeled in the presence of EGF and 0.1% Nonidet P-40 by incubation at 0°C with [γ-^{32}P]ATP (12μCi) for 2 min. The hydrolysates of gel slices containing either the 150 kDa (-●-) or the 170kDa (-O-) labeled receptor forms were analyzed sequentially on the same Sephadex G-50 column. Recovery of label applied to the column was greater than 90% for each sample. The elution characteristics of the column were determined using as molecular weight markers: blue dextran 2000 (a); carbonic anhydrase (b); myoglobin (c); cytochrome c (d); and $^{32}PO_4$ (e).

of the hydrolysed labeled material elutes where globular proteins of less than 20kDa elute, alkaline hydrolysis probably generates small phosphorylated peptides from the autophosphorylated EGF receptor forms. Hence, an increased number of alkali-labile peptide bonds in the native receptor could account for the decreased alkaline resistance of the labeled native receptor.

Alkali-generated peptides can account for the decreased alkali-resistance of the tyrosine-PO_4 only if the phosphorylated peptides are small enough to diffuse out of the gel after hydrolysis. Fig. 3 shows that autophosphorylation sites for the native receptor on tyrosines 1148 and 1173 could be found in small peptides of less than 30 amino acids because of the rapid alkaline hydrolysis of peptide bonds between residues 1137-38 and between residues 1164- 65. These peptides probably diffuse out of the gel so their tyrosine-PO_4 will appear to be alkali-labile. The other autophosphorylation site in the native receptor, tyrosine 1068, and the potential autophosphorylation sites for the 150kDa receptor form, tyrosine 845 and 992 would be found in peptides of greater than 60 amino acids. These larger peptides probably do not diffuse out of the gel as easily so their

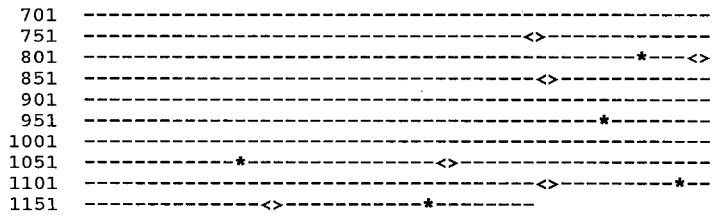

Figure 3: Sites of autophosphorylation and alkaline hydrolysis in the cytoplasmic domain of the human EGF receptor.
Based on the published (19) sequence for the human EGF receptor, positions (*) of tyrosines likely to be phosphorylated and positions (<>)of dipeptides (glycylglycine and glycy-L-serine) that are rapidly hydrolyzed by alkali are indicated. The rate constants for alkaline hydrolysis of these two dipeptides are nearly equal and about 3 times higher than for any other dipeptide (22). Tyrosines 1068, 1148, and 1173 are phosphorylated in vitro with the native receptor (9). Tyrosines 845 and 992 are potential phosphorylation sites in the 150 kDa receptor form based on published sequence criteria (20) and the similarity to the sequences around the phosphorylated tyrosines in the native receptor.

tyrosine-PO_4 would appear to be alkali-resistant. Electroblotting to nylon membranes or glutaraldehyde treatment of the gel before alkaline hydrolysis (23,24) prevents most peptides from diffusing away and could eliminate the observed alkali-sensitivity of the native receptor. Much of this sensitivity seen in Fig. 1 is explained by the proximity of two of the tyrosine-PO_4 in the native receptor to rapidly hydrolyzed alkali-labile peptide bonds and to the carboxyl terminus. A similar sensitivity is not seen with the 150kDa receptor because different tyrosines are autophosphorylated.

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