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Identification of a novel autophosphorylation site (P4) on the epidermal growth factor receptor

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Three major autophosphorylation sites are located near the *C*-terminus of the epidermal growth factor receptor, but a fourth site is repeatedly detected. We report here the purification and sequencing of a tryptic peptide containing this site, Tyr-1086. Furthermore, we demonstrate that additional phosphopeptides are observed following both partial digestion and overdigestion. Finally, we show that Tyr-1086 can be phosphorylated in intact cells.

INTRODUCTION

The cell surface receptor for epidermal growth factor (EGF) is a M_r 170000 transmembrane glycoprotein which has protein tyrosine kinase activity in its cytoplasmic domain (reviewed by Hsuan *et al.*, 1989*a*). The binding of ligands, which include EGF, transforming growth factor α and vaccinia virus growth factor, directly enhances the intrinsic kinase activity, but the mechanism of signal transduction through the plasma membrane is as yet unknown. Intramolecular activation (Koland & Cerione, 1988), receptor dissociation (Basu *et al.*, 1986) and receptor association (Yarden & Schlessinger, 1987*a*,*b*; Boni-Schnetzler & Pilch, 1987) have all been proposed as essential parts of the responses to ligand binding, and growing evidence now favours the latter process as the mechanism which causes the stimulation of kinase activity both in concentrated receptor solutions and for receptors in intact cells (Cochet *et al.*, 1988).

It is clear that the tyrosine kinase activity of the receptor is absolutely necessary for the generation of both the early and late cellular responses to EGF (reviewed by Schlessinger, 1988), but mechanistically important cytoplasmic substrates of the EGF receptor have yet to be characterized.

Autophosphorylation is known to occur at three distinct sites in the putative *C*-terminal domain of the receptor (Downward *et al.*, 1984). This domain can be removed by proteolysis (Cohen *et al.*, 1982; Gates & King, 1985; Seger *et al.*, 1988) or by mutagenesis (Clark *et al.*, 1988; Glenney *et al.*, 1988) without abolishing the ligand-dependent tyrosine kinase activity of the receptor. The effect of autophosphorylation on the tyrosine kinase activity of the EGF receptor is not clear, but recent results from both kinetic and mutagenesis studies suggest that autophosphorylation is not a necessary event for kinase activation and that it may rather allow an increase in the turnover of exogenous substrates by relieving competitive binding to the kinase domain (Honegger *et al.*, 1988*a*,*b*).

In contrast, autophosphorylation is an important regulatory mechanism in certain other tyrosine kinases, notably for example pp60$^{v\text{-}src}$ (reviewed by Hunter, 1987) and the insulin receptor (Ellis *et al.*, 1986). In these cases the regulatory autophosphorylation occurs at a tyrosine residue which is homologous to Tyr-416 of pp60$^{c\text{-}src}$ and which is conserved in all the known protein tyrosine kinase domains (Hanks *et al.*, 1988). It should also be noted that phosphorylation on Tyr-527 of pp60$^{c\text{-}src}$ negatively regulates the intrinsic tyrosine kinase activity, but this is not mediated by an autophosphorylation mechanism (reviewed by Hunter, 1987). It was therefore of interest that a fourth autophosphorylation site on the EGF receptor has been reported (Carpenter, 1987; Greenfield *et al.*, 1988; Clark *et al.*, 1988). We present here the identification of this novel site of autophosphorylation.

MATERIALS AND METHODS

Phosphopeptide mapping

A subconfluent culture of A431 cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 10 i.u. of penicillin/ml and 10 μg of streptomycin/ml, was washed twice with calcium- and magnesium-free phosphate-buffered saline (PBS) and then solubilized in lysis buffer [0.1 M-Hepes buffer, pH 7.4, containing 25 mM-benzamidine, 5 mM-EGTA, 150 mM-NaCl, 0.1% (w/v) bovine serum albumin, 5 μg of aprotinin/ml, 12.5 μg of leupeptin/ml, 50 μg of phenylmethanesulphonyl fluoride/ml and 1% (v/v) Triton X-100] at 4 °C. The lysate was cleared by centrifugation and then incubated with 1 μg of EGF/ml for 15 min at 20 °C. EGF receptors were immunoprecipitated by the addition of 4 μg of R1 monoclonal antibody (Waterfield *et al.*, 1982) and 30 μl of 50% (v/v) Protein A–Sepharose (Pharmacia) in PBS for 45 min at 4 °C. The matrix was washed with PBS containing 0.5 mM-NaCl and 0.2% (v/v) Triton X-100 and then twice with PBS containing 0.2% (v/v) Triton X-100. Phosphorylation was performed by the addition of 20 μM-[γ-^{32}P]ATP (70000 d.p.m./pmol) in 50 mM-Hepes buffer, pH 7.4, containing 0.2% (v/v) Triton X-100, 150 mM-NaCl, 2 mM-$MnCl_2$, 12 mM-$MgCl_2$ and 100 μM-Na_3VO_4 at 4 °C. After 10 min the reaction was quenched by washing three

Abbreviations used: EGF, epidermal growth factor; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.
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times with 0.1 M-EDTA, pH 8.0. Half of the matrix was treated with 1 mg of CNBr/ml in 70 % (v/v) formic acid and incubated for 16 h under N_2 at 20 °C. Following lyophilization *in vacuo*, the matrix was washed twice with water and relyophilized. Both samples were then applied to a 5–15 % SDS/polyacrylamide gel. Radiolabelled protein was detected by autoradiography and excised, digested for 24 h with 0.5 mg of trypsin/ml (202 units/mg; Cooper Biomedical) and analysed by reverse-phase h.p.l.c. essentially as described before (Hsuan *et al.*, 1989*b*).

Metabolic labelling experiments were performed by incubating subconfluent cultures of A431 cells with phosphate-free DMEM containing 5 % (v/v) DMEM, 2 % (v/v) dialysed fetal calf serum, 10 i.u. of penicillin/ml, 10 µg of streptomycin/ml and 200 µCi of $[^{32}P]P_i$/ml for 16 h. Cells were then treated with or without 1 µg of EGF/ml for 10 min at 37 °C before lysis, immunoprecipitation, tryptic digestion and analysis by h.p.l.c. as described above.

Phosphoamino acid analysis was performed by one-dimensional thin layer electrophoresis at pH 3.5 as described by Ushiro & Cohen (1980).

Purification of P4

P4 phosphopeptide was purified from a tryptic digest of a large-scale EGF receptor preparation following reduction and alkylation using the method described by Downward *et al.* (1984). The tryptic digest was applied to a Vydac RPC18 column (75 mm × 4.6 mm) and eluted with a linear gradient of acetonitrile to 50 % (v/v) in 0.08 % trifluoroacetic acid at 0.5 %/min. Fractions were counted for Čerenkov radiation and those containing P4 were purified using an Aquapore RPC4 column (200 mm × 2.1 mm) as described below.

Peptide sequence analysis

N-Terminal sequence analysis was performed using an Applied Biosystems 477A/120A pulsed gas/liquid automated sequencer.

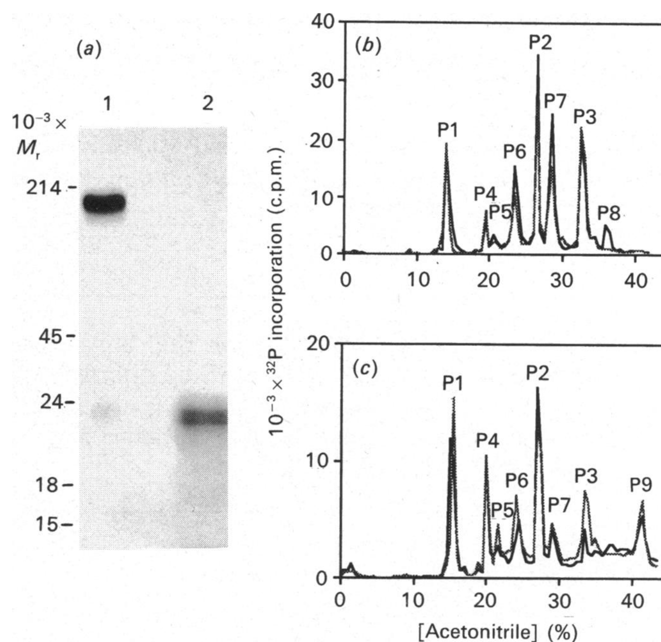

Fig. 1. Phosphopeptide mapping of the EGF receptor *C*-terminal domain

EGF receptor was purified from A431 cells by immunoprecipitation prior to phosphorylation. The preparation was then treated with or without CNBr prior to SDS/PAGE on a 5–15 % gradient gel and autoradiography (panel *a*, tracks 2 and 1 respectively). The positions of prestained standard molecular mass marker proteins are indicated. Following tryptic digestion, phosphopeptides were analysed by reverse phase h.p.l.c. as described in the text. Panels (*b*) and (*c*) show maps generated from digestion of the full length receptor (———) and the CNBr fragment (······). Digestions were performed for 24 h (*b*) and for 40 h after a further addition of 0.1 mg of trypsin/ml at 24 h (*c*). P1, P2 and P3 are labelled as described before (Downward *et al.*, 1984), and P4–P9 represent previously uncharacterized peaks.

RESULTS

The presence of an uncharacterized major phosphopeptide has been reported in tryptic digests of full-length receptor (Carpenter, 1987; Greenfield *et al.*, 1988; Clark *et al.*, 1988) and in order to identify the location of this site within the receptor we first compared phosphopeptide maps of full-length receptor with those of the *C*-terminal region. Treatment with CNBr to cleave at methionine residues was used to generate a large *C*-terminal fragment comprising amino acid residues 983–1186 as can be deduced from the complete amino acid sequence (Ullrich *et al.*, 1984) and which contains the previously identified P1, P2 and P3 autophosphorylation sites at tyrosine residues 1173, 1148 and 1068 respectively (Downward *et al.*, 1984). Solubilized EGF receptors from cultured A431 human carcinoma cells were incubated with EGF and then immunoprecipitated and phosphorylated with [γ-^{32}P]ATP. Equal aliquots were treated with or without CNBr and then analysed by SDS/PAGE. Single radiolabelled bands were detected by autoradiography in each case (Fig. 1*a*). These bands were of approximate M_r 22000 and 170000. Peptide maps of each band obtained after a 24 h digestion with trypsin were prepared and are shown in Fig. 1(*b*). It is clear from these maps that eight major phosphopeptides can be identified and that seven of these are common to both the full-length protein and the *C*-terminal CNBr fragment. Of these peptides, P1, P2 and P3 are readily identified from their previously established retention times and their levels of phosphorylation (Greenfield *et al.*, 1988; Clark *et al.*, 1988; results not shown). The fourth peptide P4 has been observed prevously (Carpenter, 1987; Greenfield *et al.*, 1988; Clark *et al.*, 1988) and here elutes between P1 and P2. The apparent level of phosphorylation of this peptide varies considerably in different experiments (compare for example Fig. 1*b* with the results of Greenfield *et al.*, 1988), but the reasons for this are unknown. The remaining phosphopeptides, numbered from P5 to P8, have also been seen in previous maps and are also variable in the amount of $[^{32}P]P_i$ incorporated in different experiments. The minor peptide P8 is the only phosphopeptide that is not found in the *C*-terminal fragment of the receptor.

In order to investigate whether partial digestion could account for the observed variability in peptide maps, further digestion of the same preparations was performed and the results are shown in Fig. 1(*c*). The major differences caused by further digestion are a relative

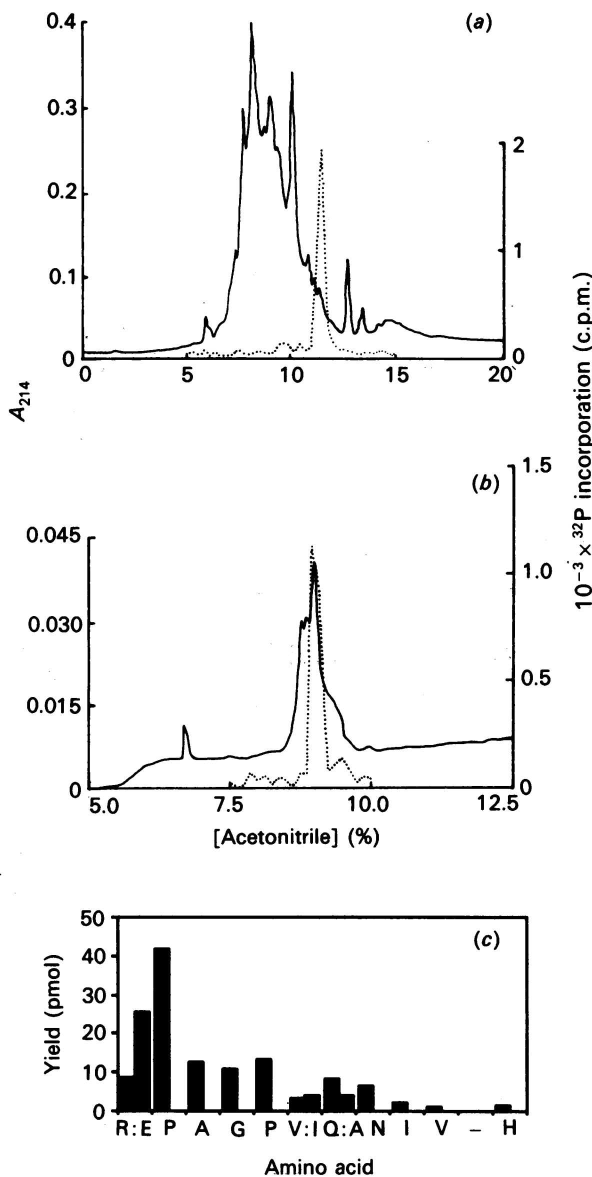

Fig. 2. Purification and *N*-terminal sequence analysis of P4

EGF receptor from A431 cells was phosphorylated, digested with trypsin and applied to a RPC18 h.p.l.c. column as described in the text. Fractions containing P4 were pooled and further separated using an Aquapore RPC4 column and developed with a linear gradient of acetonitrile [0.5% (v/v)/min] in 0.08% (v/v) trifluoroacetic acid (*a*). The radiolabelled fractions were pooled and applied to the same column but developed with 0.25% (v/v)/min acetonitrile in 0.08% (v/v) trifluoroacetic acid (*b*). The A_{214} (———) and the incorporated ^{32}P label (······) are shown. The peak radiolabelled fractions from the experiment shown in panel (*b*) were pooled and analysed by *N*-terminal sequencing as described in the text. Results from the first 12 cycles are shown in panel (*c*).

decrease in peptides P3 and P7 and a concomitant increase in peptides P4 and P9. Furthermore, fractions containing P3 could be further digested to generate P9, and fractions containing P7 could be further digested to generate P4 and P6 (results not shown). These results suggested that digestion of P3 generates P9 and digestion

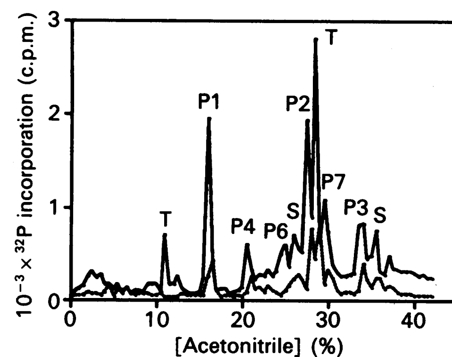

Fig. 3. Phosphopeptide mapping of the EGF receptor in intact cells

Following incubation with [^{32}P]P_i, A431 cells were treated either with (———) or without (······) EGF. Tryptic phosphopeptides of the EGF receptor were then analysed by h.p.l.c. as described in the text. Radiolabelled fractions were subsequently subjected to phosphoamino acid analysis. Peptides containing phosphotyrosine are labelled according to the results shown in Fig. 1, while S and T represent phosphopeptides containing serine and threonine respectively.

of P7 generates P4 and P6. The peptides P4, P6 and P7 therefore appeared to be derived from a previously unidentified site(s) of autophosphorylation.

Phosphopeptide P4 was purified from a large scale EGF receptor preparation (Figs. 2*a* and 2*b*) and then subjected to *N*-terminal sequence analysis. The results are shown in Fig. 2(*c*) and show clearly that Tyr-1086 is the fourth autophosphorylation site, which lies within the tryptic peptide sequence RPAGSVQNPVYHNQP-LNPAPSR (1076–1097) as deduced from the EGF receptor cDNA sequence (Ullrich *et al.*, 1984). The observed *C*-terminal location of P4 is thus consistent with the mapping results observed following cleavage with CNBr (Fig. 1*b*).

In order to examine whether or not Tyr-1086 can be phosphorylated *in vivo*, cultured A431 cells were labelled with [^{32}P]P_i and then the cells were treated with or without EGF. Phosphopeptide mapping and phosphoamino acid analysis (Fig. 3) show that EGF stimulates phosphorylation on tyrosine residues of the receptor that accounts for tryptic peptides that are consistent in retention time with P4, P6 and P7, as well as the previously characterized P1, P2 and P3 peptides (Downward *et al.*, 1984) and phosphoserine- and phosphothreonine-containing peptides (Heisermann & Gill, 1988).

DISCUSSION

The results of experiments reported here show that the previously reported fourth EGF receptor phosphopeptide is derived from a novel site of autophosphorylation. Results obtained using chemical cleavage with CNBr of the receptor showed that this peptide, termed P4, is contained within the *C*-terminal region of the receptor and the *N*-terminal sequence analysis of purified P4 peptide showed the site of phosphorylation to be Tyr-1086, which lies between the P3 (1068) and P2 (1148) sites

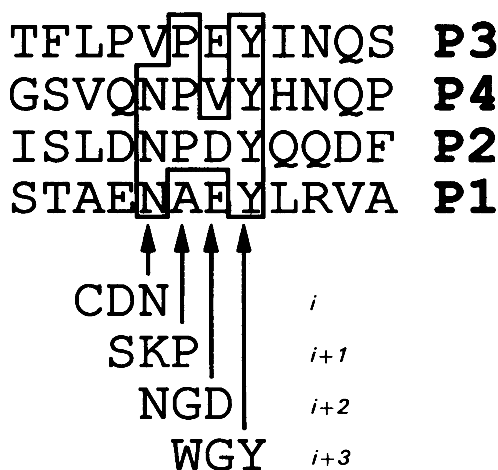

Fig. 4. Comparison of EGF receptor autophosphorylation sites

The four known autophosphorylation sites of the EGF receptor are aligned. The neighbouring amino acid residues were found to be similar to those residues predicted to occur with the highest frequency at four positions of a reverse turn as defined by Chou & Fasman (1977). These four positions are termed i, $i+1$, $i+2$ and $i+3$ from the *N*- to the *C*-terminus respectively and the most common amino acid residues found in each of these positions are shown.

(Downward *et al.*, 1984). P4 is therefore not analogous to Tyr-416 of pp60$^{c\text{-}src}$.

A comparison of the amino acid sequences surrounding the four autophosphorylation sites (Fig. 4) suggests that they are all located at reverse turns in the peptide chain, as predicted by the method of Chou & Fasman (1977). This structural motif is similar to the postulated substrate specificity of the protein tyrosine kinase LSTRA (Tinker *et al.*, 1988) and is also consistent with the substrate specificity of pp60$^{v\text{-}src}$ (Cooper *et al.*, 1984). Reverse turns are most commonly found at the surface of a protein (Kuntz, 1972) and as such may be considered accessible to binding and modification by the kinase domain. Clearly though, any precise understanding of the structural properties of autophosphorylation sites will require X-ray crystallographic analysis.

The variability in the incorporation of radiolabel into P4 may be due in part to partial digestion to give the related phosphopeptides P6 and P7. The results presented here suggest that prolonged enzymic digestion is needed to generate P4 from P6, but that P3 is concomitantly destroyed to generate P9. Further study is needed to identify the minor phosphopeptides P5 and P8, but P5 is probably also in the *C*-terminal region of the receptor (Figs. 1*b* and 1*c*).

Studies by several workers suggest that *C*-terminal autophosphorylation plays a minor role in regulating EGF receptor kinase activity (Downward *et al.*, 1985; Bertics *et al.*, 1988; Honegger *et al.*, 1988*a*,*b*), which has been interpreted in terms of competitive binding between this region and exogenous substrates to the active site of the kinase domain (Bertics & Gill, 1985). No specific role for individual autophosphorylation sites has been identified, but interestingly P4 is dephosphorylated *in vitro* by a purified human placental protein phosphotyrosine phosphatase far more slowly than the latter sites (Pallen *et al.*, 1989; C. Pallen & G. Panayotou, personal communication).

Phosphorylation on Tyr-1086 appears to occur following ligand-dependent stimulation of purified human EGF receptors that have been expressed in different cell types. These include A431 cells (Clark *et al.*, 1988; Greenfield *et al.*, 1988; results presented here), insect SF9 cells (Greenfield *et al.*, 1988) and an activated receptor in human K562 erythroleukaemic cells (J. Hsuan & H. Allen, unpublished work); as expected it is absent from a *C*-terminally truncated mutant receptor (Clark *et al.*, 1988). We have shown that this site also appears to be phosphorylated in intact A431 cells following treatment with EGF, but further studies are needed to investigate any specific role for Tyr-1086 phosphorylation.

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