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 Mr, 170000 transmembrane glycoprotein which has protein tyrosine kinase activity in its cytoplasmic domain (reviewed by Hsuan et al., 1989a). 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, 1987a,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., 1988a,b).

In contrast, autophosphorylation is an important regulatory mechanism in certain other tyrosine kinases, notably for example pp60v-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 pp60v-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 pp60v-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 phenyl-methanesulphonyl 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-32P]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-MnCl2, 12 mM-MgCl2 and 100 μM-Na3VO4 at 4 °C. After 10 min the reaction was quenched by washing three
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 N₂ 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/ml; Cooper Biomedical) and analysed by reverse-phase h.p.l.c. essentially as described before (Hsuan et al., 1989b).

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, 0.1 i.u. of penicillin/ml, 10 μg of streptomycin/ml and 200 μCi of [32P]Pi/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, trypptic 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 trypptic digest of a large-scale EGF receptor preparation following reduction and alkylation using the method described by Downward et al. (1984). The trypptic 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 Cerenkov 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.

**RESULTS**

The presence of an uncharacterized major phosphopeptide has been reported in trypptic 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-32P]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. 1a). These bands were of approximate Mr, 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 previously (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. 1b 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 [32P]P 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...
Epidermal growth containing P3 could be P4 in increase in decrease that suggested and fractions of EGF receptor preparation (Figs. 2a and 2b) 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 RPAKSVQNPVYHNQP-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. 1b).

In order to examine whether or not Tyr-1086 can be phosphorylated in vivo, cultured A431 cells were labelled with [32P]P, 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.
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<sup>src</sup>.

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<sup>src</sup> (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. 1b and 1c).

Studies by several workers suggest that C-terminal autophosphorylation plays a minor role in regulating EGF receptor kinase activity (Downward et al., 1985; Bertsics et al., 1988; Honegger et al., 1988a,b), which has been interpreted in terms of competitive binding between this region and exogenous substrates to the active site of the kinase domain (Bertsics & 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 follow 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.

Fig. 4. Comparison of EGF receptor autophosphorylation sites

REFERENCE

Greenfield, C., Patel, G., Clark, S., Jones, N. & Waterfield, M. D. (1988) EMBO J. 7, 139-146
Kuntz, J. D. (1972) J. Am. Chem. Soc. 94, 4009-4012

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