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Kinetic parameters of the protein tyrosine kinase activity of EGF-receptor mutants with individually altered autophosphorylation sites

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Epidermal growth factor (EGF)-receptor mutants in which individual autophosphorylation sites (Tyr1068, Tyr1148 or Tyr1173) have been replaced by phenylalanine residues were expressed in NIH-3T3 cells lacking endogenous EGF-receptors. Kinetic parameters of the kinase of wild-type and mutant receptors were compared. Both wild-type and mutant EGF-receptors had a K_m-(ATP) 1-3 μM for the autophosphorylation reaction, and a K_m(ATP) of 3-7 μM for the phosphorylation of a peptide substrate. These are similar to the K_m(ATP) values reported for EGF-receptor of A431 cells. A synthetic peptide representing the major *in vitro* autophosphorylation site Tyr1173 of the EGF-receptor (KGSTAENAEYLRV) was phosphorylated by wild-type receptor with a K_m of 110-130 μM, and the peptide inhibited autophosphorylation with a K_i of 150 μM. Mutant EGF-receptors phosphorylated the peptide substrate with a K_m of 70-100 μM. A similar decrease of K_m (substrate) was obtained when the phosphorylation experiments were performed with the commonly applied substrates angiotensin II and a peptide derived from c-*src*. The K_m of angiotensin II phosphorylation was reduced from 1100 μM for wild-type receptor to 890 μM for mutant receptor and for c-*src* peptide from 1010 μM to 770 μM respectively. The V_{max} of the kinase was dependent on receptor concentration, but was not significantly affected by the mutation. Analogs of the Tyr1173 peptide in which the tyrosine residue was replaced by either a phenylalanine or an alanine residue also inhibited autophosphorylation with K_i of 650-750 μM. These analyses show that alterations of individual autophosphorylation sites do not have a major effect on kinase activity. The V_{max} of the reaction is not affected, and the small increase in substrate affinity following autophosphorylation may be due to removal of competitive inhibition by intrinsic substrate sites.

Key words: epidermal growth factor/EGF-receptor kinase/ Michaelis-Menten kinetics

Introduction

Many of the known protein tyrosine-kinases catalyze the phosphorylation of both external substrates and of multiple intrinsic sites. In some instances, autophosphorylation has been shown to play a role in regulating the enzymatic activity of kinases, and several oncogene products derived from cellular tyrosine protein kinases show altered autophosphorylation sites compared to their normal counterparts (reviewed in Yarden and Ullrich, 1988). A consensus tyrosine residue within the kinase domain was shown to be involved in the activation of insulin receptor kinase (Ellis *et al.*, 1985), $pp^{60c\text{-}src}$ kinase (Cartwright *et al.*, 1987; Kimiecik *et al.*, 1987) and $pp^{120gag\text{-}fps}$ kinase (Meckling-Hansen *et al.*, 1987). These kinases are activated by autophosphorylation. Substitution of the consensus tyrosine by a phenylalanine residue by site-directed mutagenesis critically impaired kinase activity. In the case of insulin receptor kinase, it has been shown that autophosphorylation increases the V_{max} of the kinase activity, maintaining the receptor kinase in an activated state even in the absence of ligand binding (Rosen *et al.*, 1985).

The identified autophosphorylation sites of epidermal growth factor receptor (EGF-R) are located at the C-terminal end of the receptor molecule at positions 1068, 1148 and 1173 respectively (Downward *et al.*, 1984a). Phosphorylation of Tyr845, corresponding to the consensus tyrosine of autophosphorylation regulated kinases, has not yet been demonstrated for the EGF-R. The v-*erbB* oncogene product which is derived from the avian EGF-R lost two of the three autophosphorylation sites (Downward *et al.*, 1984b). It was reported that autophosphorylation of EGF-R increased (Bertics and Gill, 1985) or had no effect (Downward *et al.*, 1985) on the receptor kinase activity towards exogenous substrates.

In order to test the role of receptor autophosphorylation in regulating the various responses of EGF, we have utilized *in vitro* site-directed mutagenesis to generate EGF-R mutants with individually altered autophosphorylation sites. The accompanying report (Honegger *et al.*, 1988) describes the generation of the mutant receptors, their expression in NIH-3T3 cells lacking endogenous EGF-R, and their biological properties. It appears that none of the vital functions of the EGF-R were impaired by the loss of individual autophosphorylation sites. Moreover, cells expressing mutant receptors were slightly more mitogenically responsive to low concentrations of EGF than cells expressing wild-type receptors. To test whether this is due to alteration in the enzymatic activities of the mutant receptors, we have analyzed the kinetic parameters of the kinase activities of wild-type and mutant receptors. Here we show that autophosphorylation of EGF-R does not have a large effect on its kinase activity. The V_{max} of the phosphorylation reaction is not affected, and the small increase in substrate affinity upon autophosphorylation can be explained by removal of competitive inhibition by intrinsic substrate sites.

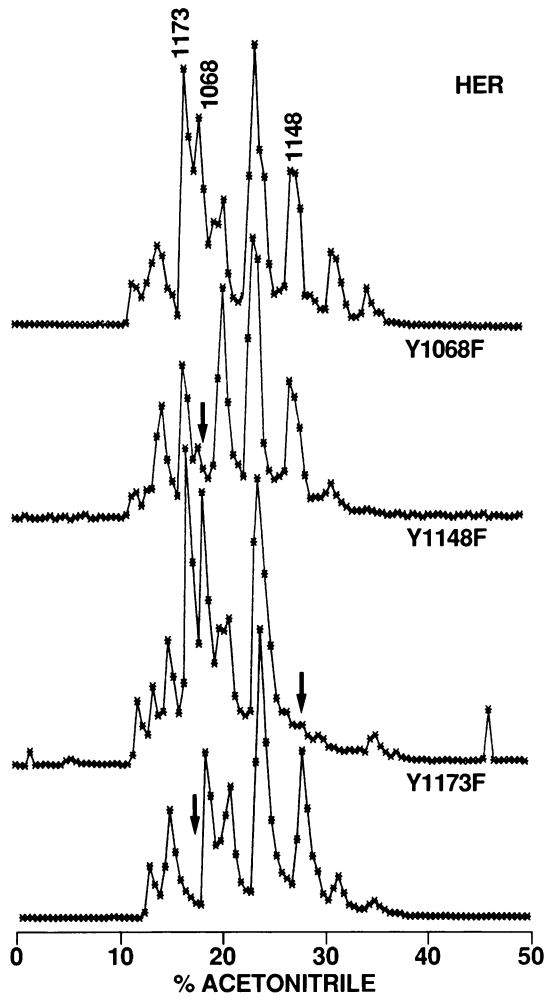

Fig. 1. Phosphopeptide maps of phosphorylated wild-type or mutant EGF-R analysed by HPLC. Lysates of cells expressing either wild-type or mutant EGF-R (Y1068F, Y1148F or Y1173F) were incubated with 0.5 μg/ml EGF for 10 min at 30°C, then immunoprecipitated with monoclonal antibodies against EGF-R (mAb 108.1). The washed immunoprecipitates were incubated with 10 μCi of [γ-^{32}P]ATP in the presence of 5 mM $MnCl_2$ for 20 min at room temperature and analyzed on a 7% SDS–polyacrylamide gel. The bands corresponding to the phosphorylated EGF-R were excised and digested with TPCK-trypsin at 37°C for 12 h. The tryptic peptides were separated by HPLC on a C_{18} column using a 0–30% gradient of acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min. Fractions of 0.5 ml each were collected and counted in a scintillation counter.

Results

In vitro site-directed mutagenesis was used to substitute individual autophosphorylation tyrosine sites of the EGF-R by phenylalanine residues. Wild-type or mutant receptor cDNA constructs were transfected and shown to be expressed in NIH-3T3 cells devoid of endogenous EGF-R. Construction of the expression plasmids, transfections, characterization of cell lines which express mutant receptors and their biological properties are described in the accompanying manuscript (Honegger *et al.*, 1988).

Wild-type and mutant receptors were immunoprecipitated from cell lysates using a monoclonal antibody against the human EGF-R (mAb 108.1) bound to protein A–Sepharose. For ligand activation, the cell lysates were incubated with 1 μg/ml EGF prior to immunoprecipitation. *In vitro* phosphorylation experiments were performed by incubating the immunoprecipitated EGF-R with [γ-^{32}P]ATP and with the peptide substrate. The reaction products were separated by SDS–PAGE. The relative concentrations of the different receptor mutants were determined by immunoblotting the samples with anti-peptide antibodies against EGF-R (RK2) and by quantitating the radioactive content of ^{125}I-labeled protein A bound to the immunoblots.

Autophosphorylation

Wild-type EGF-R and the various mutants were autophosphorylated *in vitro* with [γ-^{32}P]ATP, analyzed by SDS–PAGE and digested with TPCK (L-1-tosylamide-2-phenylethyl-chloromethyl ketone)-trypsin. The tryptic peptides were eluted from the gel and analyzed by reverse-phase HPLC, using an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) to elute the peptides from a C_{18} column. Phosphopeptides were detected by counting the column fractions in a β-counter. Receptor immunoprecipitated from transfected NIH-3T3 cells expressing human wild-type EGF-R produced a peptide map identical to that of EGF-R immunoprecipitated from A431 cells (data not shown). Each of the autophosphorylation site mutants lacked one major peak as compared to wild-type EGF-R (Figure 1). EGF-R mutants Y1173F and Y1173S showed identical phosphopeptide maps, both lacking the peak which contains phosphorylated Tyr1173. A deletion mutant of EGF-R lacking 63 amino acids at the C terminus (Livneh *et al.*, 1986) and thus lacking both Tyr1173 and Tyr1148 lost the appropriate peaks containing these phosphorylated residues, while all the other peaks in the map remained (not shown), further confirming the assignment of Y1173 and Y1148. These results show that each of the mutant EGF-R lacks one particular autophosphorylation site and that the *in vitro* phosphorylation of the remaining sites is not affected by the loss of one particular autophosphorylation site.

Specific activity

The relative concentration of EGF-R was determined by immunoblotting with anti-EGF-R antibodies (RK2) and ^{125}I-labeled protein A. The amount of [^{125}I]protein A bound to the immunoblots showed a linear dependence on the amount of immunoprecipitate loaded on the gel (Figure 2A and B). The slope of resulting lines is therefore a direct measure of the relative concentration of EGF-R in the different immunoprecipitates. Autophosphorylation activity (c.p.m. incorporated/min) also showed linear dependence on the amount of immunoprecipitated EGF-R (Figure 2A and C), indicating that in the immunoprecipitate the concentration of the EGF-R does not affect its specific activity. Plotting phosphorylation activity against immunoblot signal revealed a linear dependence for each mutant receptor with a slope determined by the specific activity of the receptor molecule (Figure 2D). As expected, the different EGF-R mutants showed different specific activities for autophosphorylation (Table I). However, the specific activity of each mutant receptor was correlated with the initial receptor concentration in the cell lysate. When the experiments were repeated with cell lysates which were diluted to approximately the same receptor concentration, the specific activities of the mutant receptors converged to similar values. This result is consistent with the idea that the activation of the EGF-R kinase involves an intermolecular process (Yarden and Schlessinger, 1987).

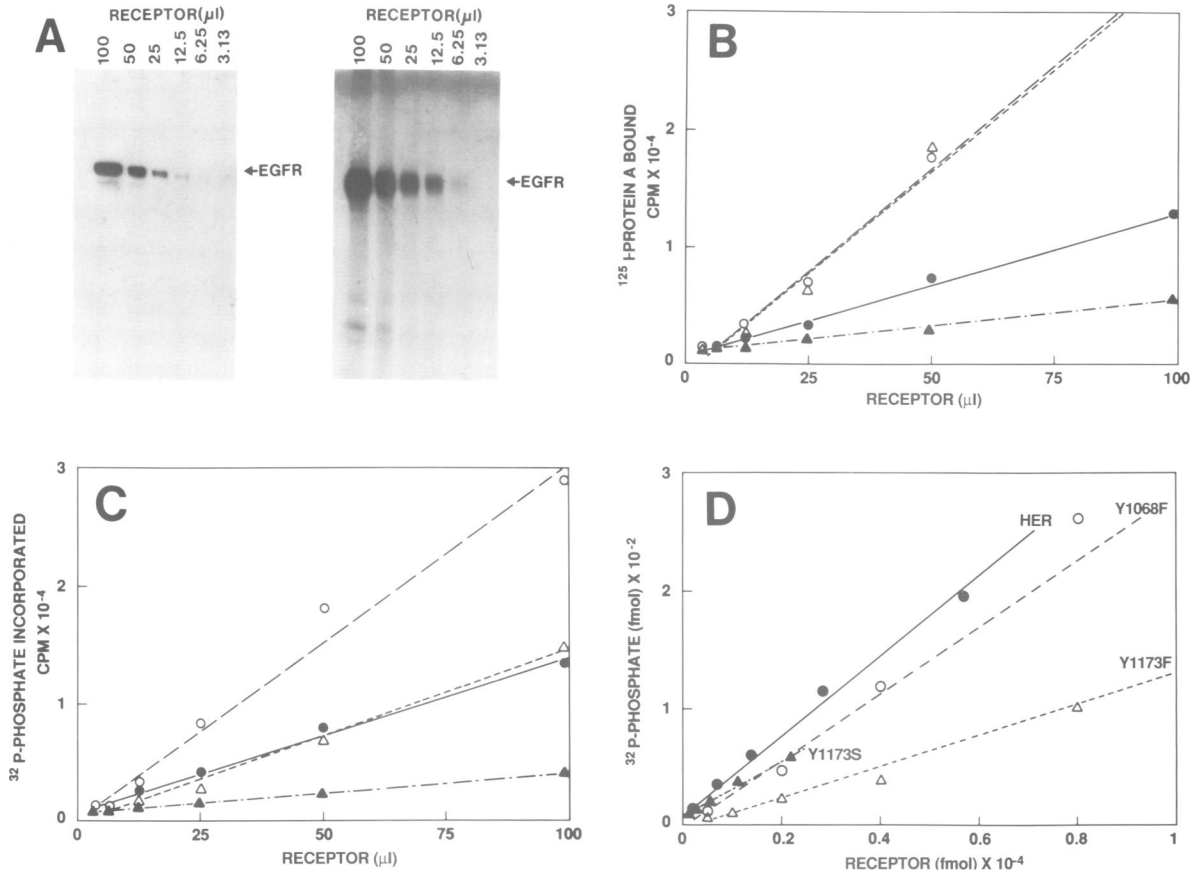

Fig. 2. Specific activity of wild-type or mutant receptors for autophosphorylation. (**A**) Equal amounts of immunoprecipitated receptor were either analyzed by SDS−PAGE and immunoblotting with anti-EGF-R antibodies RK2 (left) or autophosphorylated under initial rate conditions and separated from free [γ-^{32}P]ATP by SDS−PAGE (right). (**B**) The amount of [^{125}I]protein A bound to the immunoblots was quantitated by cutting out the radioactive bands from the blots and counting them in a γ-counter. Radioactivity was plotted as a function of the amount of immunoprecipitate loaded into the corresponding slot of the gel. The slope of each line is directly proportional to the amount of receptor protein per ml of immunoprecipitate. (**C**) The amount of [^{32}P]phosphate incorporated into a given amount of immunoprecipitate per min under initial rate conditions for phosphorylation was quantitated by cutting out the bands of phosphorylated EGF-R and counting Cerenkov radiation in a β-counter. The radioactivity incorporated per min was plotted against the amount of immunoprecipitate used in the reaction. The slope of each line is proportional to the kinase activity of the EGF-R per μl of immunoprecipitate. (**D**) The autophosphorylation signal for each amount of immunoprecipitate was plotted as a function of the immunoblot signal for the same amount of immunoprecipitate. The relative specific activity of each mutant was deduced from the slope of the line compared to the slope of the line for the wild-type EGF-R since the immunoblot signal is directly proportional to the absolute receptor concentration. The specific activity for ATP was determined by absorbing a known amount of ATP solution into a lyophilized piece of gel and counting the Cerenkov radiation emitted per fmol of ATP to obtain an estimate of the absolute amounts of [^{32}P]phosphate incorporated in the autophosphorylation reaction. 100 μl of immunoprecipitate of wild-type receptor corresponded to the amount of receptor obtained from $\sim 10^7$ cells (300 000 receptors/cell). The maximal amount of receptor per 1 ml of immunoprecipitate is therefore $\sim$17 fmol. In this analysis, we assume a 100% recovery of the receptor and, therefore, it represents an upper limit for receptor concentration rather than an accurate determination. Relative concentrations for the different mutants were obtained from the ratio of the slopes of amount of [^{125}I]protein A bound versus ml of immunoprecipitate. This was used to estimate the relative specific activity of each receptor mutant.

Substrate phosphorylation

A synthetic peptide representing the major autophosphorylation site of EGF-R Y1173 (KGSTAENAEYLRV) was used as a substrate for the EGF-R kinase. This peptide was readily phosphorylated by the EGF-R kinase with a K_m of 110−130 μM. EGF increased the V_{max} of the reaction without affecting the K_m of the peptide (Figure 3). At a peptide concentration of 1 mM, substrate phosphorylation was three times faster than EGF-R autophosphorylation determined in the absence of peptide substrate (Table I). The autophosphorylation of wild-type or mutant EGF-R with unlabeled ATP greatly diminished subsequent receptor autophosphorylation with [γ-^{32}P]ATP. However, the specific activities of wild-type or mutant receptors toward saturating concentrations of peptide substrate were not significantly affected by autophosphorylation with unlabeled ATP (Table I). Similarly, the mutations did not have a significant effect on the specific activities of the unphosphorylated receptor molecules (Figure 4A).

Michaelis−Menten (K_m) constants for ATP and for peptide substrate phosphorylation

The Michaelis−Menten constant K_m for ATP is dependent on the type and concentration of divalent cations added to the phosphorylation reaction mixture. In 5 mM $MnCl_2$, the K_m(ATP) for autophosphorylation was 1−3 μM and the K_m(ATP) for peptide substrate phosphorylation was 3−7 μM (Table II; Figure 4B). These values are in good accord with the K_m of 3−5 μM which was determined for the EGF-R of A431 cells (Erneux *et al.*, 1983; Weber *et al.*, 1984). Wild-type EGF-R phosphorylated the peptide substrate with a K_m of 110−130 μM, while the mutants showed a K_m of 70−100 μM for peptide substrate phosphorylation (Table II; Figure 4C).

Table I. Relative specific activities of wild-type and mutant EGF-R for autophosphorylation and phosphorylation of exogenous substrates

EGF-R mutants	Control	After auto-phosphorylation with unlabeled ATP	In the presence of substrate 0.33 mM	1 mM
Autophosphorylation				
Wild-type	100[a]	10	70	20
Y1068F	95	7	50	40
Y1148F	50	–[b]	70	–
Y1173F	95	7	40	20
Y1173S	70	–	70	25
CD63	50	5	–	20
Peptide substrate phosphorylation				
Wild-type	250	210		
Y1068F	270	210		
Y1148F	180	–		
Y1173F	340	320		
Y1173S	210	190		
CD63[c]	390	250		

[a]Autophosphorylation of wild-type EGF-R in the absence of peptide was assigned a relative specific activity of 100 units, corresponding to 63 c.p.m. ^{32}P incorporated per min per 1 μl immunoprecipitate or 14 400 c.p.m. ^{32}P incorporated per min by the amount of receptor giving a signal of 100 000 c.p.m. on an immunoblot with EGF-R antibodies. The specific activities of mutant receptors for peptide phosphorylation are relative to this standard. The relative specific activities correlated with the concentration of EGF-R in the cell lysate prior to immunoprecipitation. This is in accord with the notion that the activation of the kinase function involves an intermolecular process (Yarden and Schlessinger, 1987). EGF enhanced the V_{max} of the kinase activity of wild-type and mutant receptors in the range of 1.5- to 2-fold.
[b]In certain experiments the signal was too low for reliable quantitation.
[c]CD63 is an EGF-R mutant lacking 63 amino acids at the C terminus and thus missing the autophosphorylation sites Y1148 and Y1173.

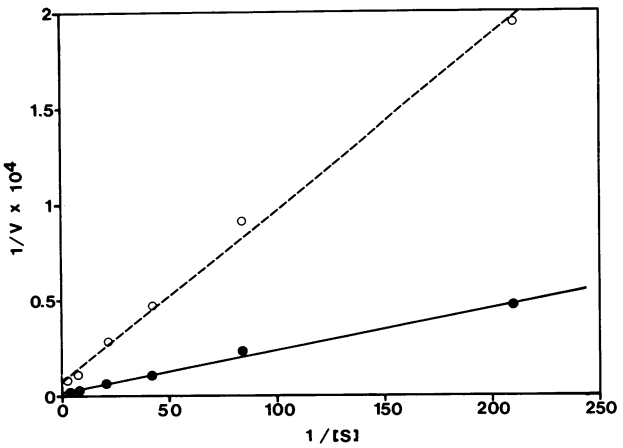

Fig. 3. Lineweaver–Burk plot of peptide substrate phosphorylation by wild-type EGF-R. Lysates of cells were incubated for 10 min at 30°C with (—●—) or without (--○--) 1 μg/ml EGF and the EGF-R was immunoprecipitated with mAb 108.1. Reciprocal velocity of peptide phosphorylation was plotted as a function of reciprocal substrate concentration.

Angiotensin II (DRVYIHPF) and a peptide representing the autophosphorylation site of c-*src* (RRLIEDAEYAARG, Pike *et al.*, 1982) were used as control substrates to determine whether the observed changes in K_m were a general phenomenon or unique to the peptide representing the autophosphorylation site of EGF-R. The results presented in Figure 6 show that both peptides had a similar effect on the autophosphorylation of EGF-R. However, their K_m were ~10-fold lower than the K_m values of the peptide representing the autophosphorylation site of the EGF-R (Figure 6). The wild-type receptor phosphorylated angiotensin II with a K_m of 1100 μM and the mutant receptor Y1068F with a K_m of 890 μM. The c-*src* derived peptide was phosphorylated with a K_m of 1010 μM by the wild-type receptor and with a K_m of 770 μM by the Y1068F mutant EGF-R. Since no qualitative differences were found between autophosphorylation site peptide and the unrelated peptide substrates, the rest of the experiments were performed with the best substrate, namely, with the peptide derived from the autophosphorylation site of EGF-R.

Effect of peptide substrate on autophosphorylation of wild-type and mutant EGF-R

The peptide substrate acted as a competitive inhibitor of receptor autophosphorylation. Thus, we have also explored the effect on the autophosphorylation of the EGF-R of peptides which contain either phenylalanine or alanine instead of a tyrosine residue (Figure 5). The authentic peptide substrate containing tyrosine inhibited autophosphorylation of EGF-R with an inhibition constant K_i of 150 μM, a value close to the K_m of 110–130 μM which was measured for this reaction. The modified peptides which cannot be phosphorylated since they contain phenylalanine or alanine instead of tyrosine also inhibited autophosphorylation of EGF-R with a K_i of 650–750 μM.

Discussion

Autophosphorylation has been reported to play a regulatory role in a number of protein tyrosine kinases (reviewed in Yarden and Ullrich, 1988). It was shown that the kinase activities of the insulin receptor (Ellis *et al.*, 1985), $pp^{60c\text{-}src}$ (Cartwright *et al.*, 1987; Kmiecik *et al.*, 1987; Piwnica-Worms *et al.*, 1987) and $pp^{120gag\text{-}fps}$ (Meckling-Hansen *et al.*, 1987) were greatly diminished upon substitution of a consensus tyrosine in the kinase domain by a phenylalanine residue. It was also shown that autophosphorylation of insulin receptor leads to an increased V_{max} of the kinase, suggesting a regulatory role for receptor autophosphorylation (Rosen *et al.*, 1983). The identified autophosphorylation sites of the EGF-R are located in the C-terminal end of the receptor molecule in positions 1068, 1148 and 1173 respectively (Downward *et al.*, 1984). In order to explore the role of EGF-R autophosphorylation we have utilized *in vitro* site-directed mutagenesis to prepare EGF-R mutants with altered autophosphorylation sites. The expression constructs encoding either wild-type or mutant EGF-R were transfected into NIH-3T3 cells lacking endogenous EGF-R, which after selection with antibiotics and cloning were shown to express either wild-type or the mutant receptors. The transfected cells expressing mutant receptors bind EGF and become mitogenically responsive to the growth factor (Honegger *et al.*, 1988). EGF was also able to stimulate the autophosphorylation of the mutant receptors in living cells, indicating that mutant receptors have an active protein tyrosine kinase and that modification of individual autophosphorylation sites does not impair the autophosphorylation of the remaining sites. Comparison of phosphopeptide maps of tryptic digests of *in vitro* phosphorylated wild-type or mutant receptors

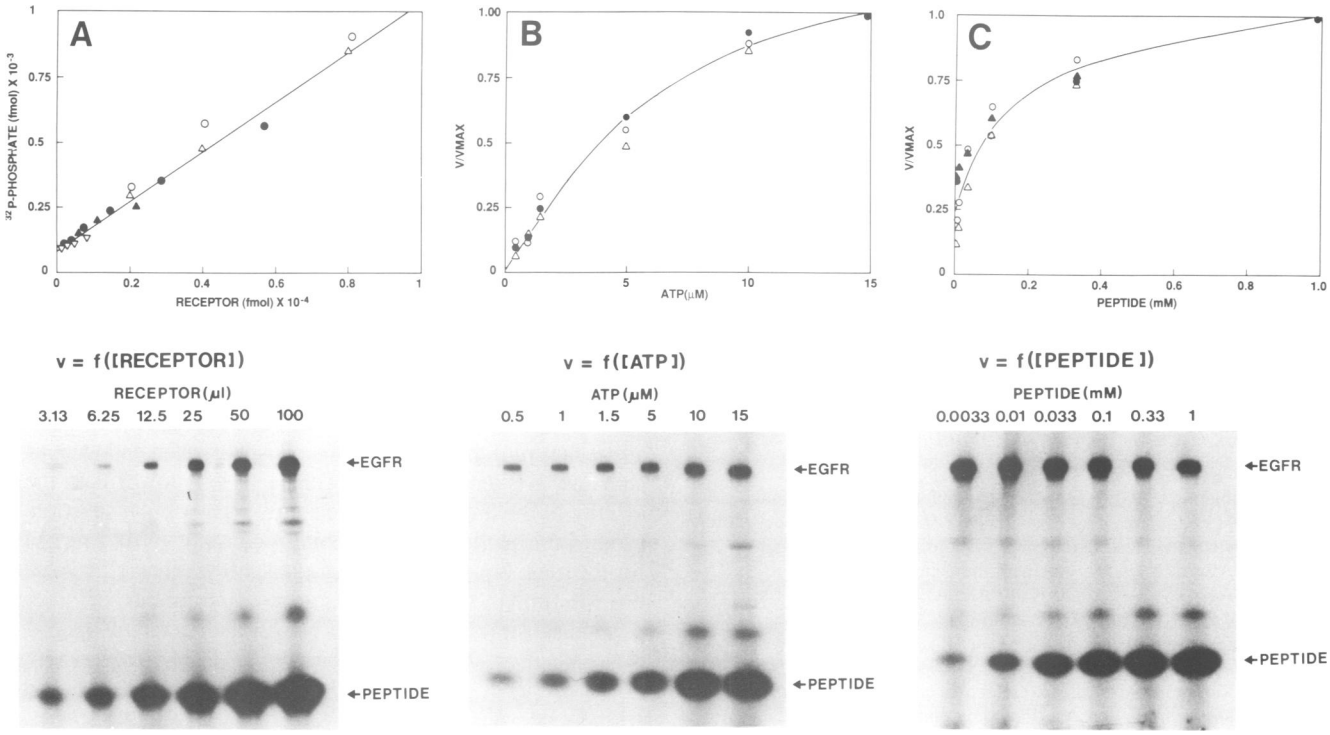

Fig. 4. Kinetic parameters for peptide phosphorylation. A synthetic peptide representing a sequence around the major autophosphorylation site of EGF-R Y1173 was used as a substrate for the determination of the kinetic parameters of the kinases of wild-type and mutant receptors. The phosphorylation reaction was allowed to proceed for 1 min at 4°C before it was stopped. The phosphorylated receptors, phosphorylated peptide substrate and free [γ-^{32}P]ATP were separated by SDS–PAGE. The amount of [^{32}P]phosphate incorporated was quantitated by excising the radioactive band and counting their Cerenkov radiation in a β-counter. HER (●); Y1068F (○); Y1173F (△); Y1173S (▲); and CD63 (▽). **(A)** Peptide phosphorylation as a function of receptor concentration. Different amounts of immunoprecipitate were used to phosphorylate peptide substrate under conditions where both the concentration of ATP (15 μM) and peptide concentration (1 mM) were well above the respective K_m values. The receptor concentrations were determined by an immunoblotting procedure as described in the legend to Figure 2. **(B)** Peptide phosphorylation as a function of ATP concentration. Equal aliquots of immunoprecipitated receptor were used to phosphorylate the peptide substrate (200 μM) in the presence of increasing concentrations of ATP. The specific activities of the different mutants diluted to approximately equal receptor concentration prior to immunoprecipitation are the same as in (A). Therefore, V/V_{max} was plotted against the concentration of ATP to compare directly between mutant receptors. **(C)** Peptide phosphorylation as a function of peptide concentration. Equal amounts of immunoprecipitated receptors were used to phosphorylate different concentrations of peptide substrate at 10 μM ATP.

Table II. Michaelis–Menten constants (K_m)[a] of the protein tyrosine kinases of wild-type and mutant EGF-R for ATP and for peptide substrate

	EGF	K_m(ATP) for autophosphorylation (μM)	K_m(ATP) for peptide phosphorylation (μM)	K_m(peptide) for peptide phosphorylation (μM)
Wild-type	+	1.0–2.5	4.0–8.0	110
	–	1.0–3.0	5.5–13	130
Y1068F	+	1.0–1.5	4.0	70
	–	2.0	2.5–7.0	100
Y1148F	+	0.5–1.0	1.0–2.5	–
	–	1.0–2.0	1.0–2.5	–
Y1173F	+	0.5–1.0	4.0–5.0	80
	–	1.0–1.5	7.0	90
Y1173S	+	1.5–2.5	2.5–7.5	70
	–	1.0–3.0	3.5–7.5	100

[a]K_m values were determined by linear regression in the Lineweaver–Burk representation for the dependence of the reaction velocity on substrate concentration.

confirmed the loss of one individual autophosphorylation site in each mutant, and further demonstrated that autophosphorylation of the remaining sites is not affected by the mutations (Figure 1). Except for the loss of individual autophosphorylation sites, no qualitative differences were found between wild-type and mutant receptors. The mutant receptors were able to perform all the biological functions of normal EGF-R. However, cells expressing mutant receptors appeared to be slightly more mitogenically responsive to low concentration of EGF than cells expressing similar amounts of wild-type EGF-R (Honegger *et al.*, 1988).

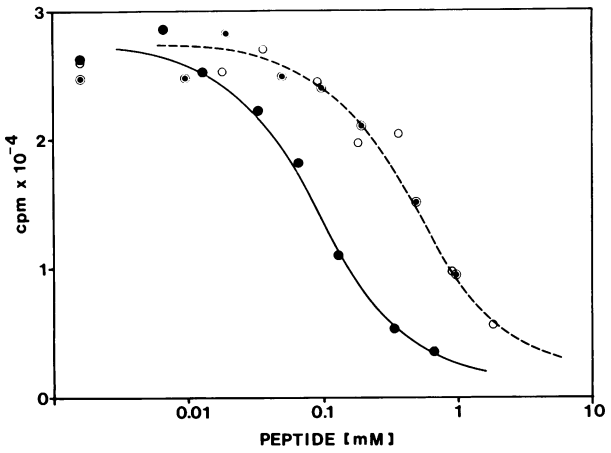

Fig. 5. Inhibition of EGF-R autophosphorylation by substrate and analog peptides. Immunoprecipitates of EGF-stimulated wild-type EGF-R were incubated with different concentrations of peptide substrate KGSTAENAEYLRV (—●—) or with the peptides KGSTAENAEFLRV (--⊙--) or KGSTAENAELRV (--○--) for 15 min at 4°C before [γ-^{32}P]ATP and $MnCl_2$ were added. The reaction was allowed to proceed for 2 min under shaking and was then stopped by addition of 3 × SDS sample buffer and heating to 95°C for 4 min. Receptor and peptide were separated by gel electrophoresis and phosphate incorporation determined by cutting out radioactive bands after autoradiography and counting Cerenkov radiation. [^{32}P]Phosphate incorporation into the EGF-R was plotted as a function of peptide concentration on a logarithmic scale.

To test the effect of receptor autophosphorylation on the kinase activity of the EGF-R we have analyzed the kinetic properties of the protein tyrosine kinase of the mutant receptors and compared them to the properties of wild-type EGF-R. We have shown that both wild-type and mutant EGF-R had a K_m for ATP of 1−3 μM for autophosphorylation and a K_m(ATP) of 3−7 μM for peptide substrate phosphorylation. These K_m values are similar to those reported for the EGF-R of A431 cells (Erneux *et al.*, 1983; Weber *et al.*, 1984).

The peptide substrate used in this study is a synthetic peptide representing the major autophosphorylation site of EGF-R (Tyr1173). Wild-type EGF-R phosphorylated the peptide substrate with a K_m of 110−130 μM while mutant EGF-R phosphorylated the peptide substrate with a K_m of 70−100 μM. Hence, the K_m of the mutant receptors for peptide substrate phosphorylation is slightly reduced as compared to the K_m of wild-type EGF-R. Two additional commonly used peptide substrates were employed in this study. These peptide substrates were used to examine the generality of effects found with the peptide representing the autophosphorylation site of EGF-R. Wild-type EGF-R phosphorylated the peptide substrate angiotensin II with a K_m of 1100 μM and the mutant receptor Y1068F with a K_m of 890 μM. Similar results were obtained with a synthetic peptide representing an autophosphorylation site of c-*src*. Wild-type EGF-R phosphorylated this peptide with a K_m of 1010 μM and the mutant EGF-R Y1068F phosphorylated this peptide with a K_m of 770 μM. The higher affinity of the EGF-R derived peptide substrate made it more suitable for kinetic analysis. This is because higher saturation of the receptor could be achieved at lower substrate concentrations which did not influence the viscosity of the reaction mixture. Hence, most of the experiments were performed with the superior substrate.

It was previously demonstrated that the kinetics of peptide substrate phosphorylation by the EGF-R kinase follows a sequential ordered bi-bi mechanism. In this process the binding of the peptide is followed by ATP binding. The phosphopeptide is released first and ADP is the last product to be released (Erneux *et al.*, 1983). Exogenous substrates competitively inhibit receptor autophosphorylation (Cassel *et al.*, 1983). It is possible that exogenous substrate peptides and the endogenous phosphorylation sites compete for the substrate binding region in the kinase domain of the EGF-R, and that the unphosphorylated sites within the receptor therefore competitively inhibit the phosphorylation of exogenous substrates. The 2- to 3-fold enhanced kinase activity of the phosphorylated versus the non-phosphorylated EGF-R at low substrate concentration is consistent with such a mechanism if the affinity of the phosphorylated endogenous sites towards the active site of the kinase is lower than the affinity of the unphosphorylated sites. The peptide substrate used in this study had an inhibition constant K_i of 150 μM for the inhibition of EGF-R autophosphorylation. This value is in a similar range to the inhibition constants of other peptide substrates of tyrosine kinases: angiotensin II (K_m = 800 μM, K_i = 700 μM, Weber *et al.*, 1984); gastrin (K_m = 53−87 μM, K_i = 60 μM, Baldwin *et al.*, 1983) and *src*-peptide (K_m = 600 μM, K_i = 1 μM, Weber *et al.*, 1984). In all cases the K_m values for peptide phosphorylation are close to the corresponding K_i value for inhibition of autophosphorylation. Analogs of the peptide substrate used in this study containing phenylalanine or alanine in place of tyrosine also inhibited autophosphorylation, but with a K_i (650−750 μM) higher than the K_i of the tyrosine-containing peptides (K_i = 150 μM). It is not clear yet whether the phosphorylated form of the tyrosine-containing peptide is able to inhibit autophosphorylation. It appears from the data of Bertics and Gill (1985) that if it does inhibit autophosphorylation, its K_i should be higher than the K_i of the unphosphorylated substrate. Recently, Bertics *et al.* (1988) reported that a mutant EGF-R showed decreased biological and enzymatic activity. However, the EGF-R mutant described in this study contains in addition to the altered autophosphorylation site Tyr1173 an additional mutation in which Thr654 was replaced by an alanine residue. Moreover, their 'wild-type' EGF-R contains also the same mutation at Thr654. Hence, the difference in the results could be attributed to these or additional mutations in their EGF-R constructs.

Competitive inhibition by intramolecular sites in the EGF-R kinase should increase the apparent K_m of the peptide substrate without affecting the V_{max} of the process. Increasing the K_i of the competing sites by phosphorylation or by replacing tyrosine with other amino acids would decrease the apparent K_m for the peptide substrate and, therefore, increase the activity of the receptor at low substrate concentrations. At high concentration of peptide substrate the activity of the kinase should not be affected by the phosphorylation state or by mutations of the autophosphorylation sites. If phosphorylation of 2−3 sites leads to 2- to 3-fold increase of apparent autophosphorylation activity (Bertics and Gill, 1985), phosphorylation of a single site would correspond to a 40−70% increase in activity. Similarly, the effect of replacing one of the tyrosine residues by phenylalanine should yield an effect of comparable magnitude to the phosphorylation of a single site. Indeed,

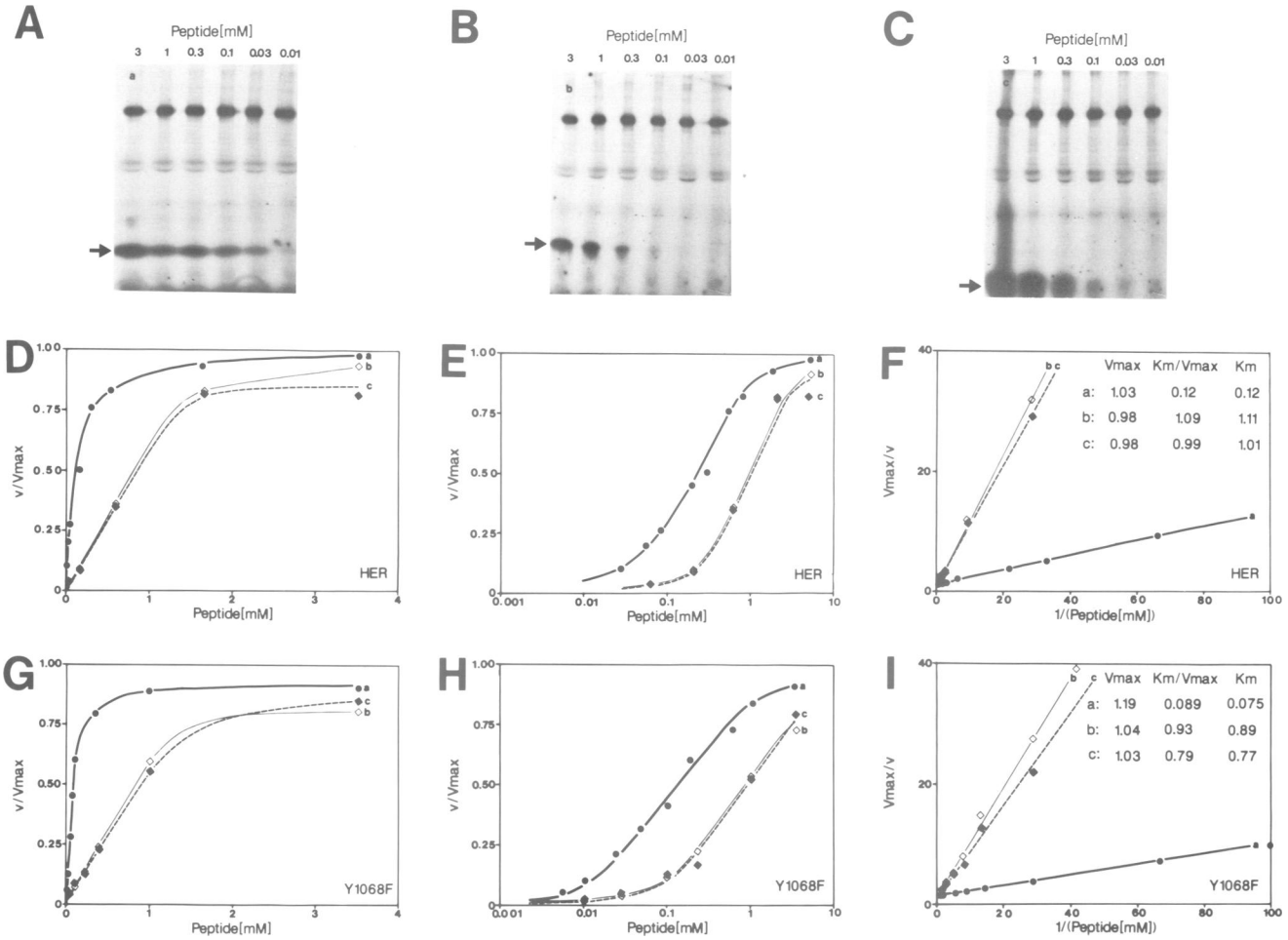

Fig. 6. Phosphorylation of three substrate peptides by wild-type EGF-R and by the Y1068F EGF-R mutant. Peptide representing autophosphorylation site of EGF-R Y1173 (KGSTAENAEYLRV), peptide a; angiotensin II (DRVYIHPF), peptide b; and the c-*src*-derived site peptide (RRLIEDAEYAARG), peptide c; were used as substrates for phosphorylation by the wild-type EGF-R and by the Y1068F EGF-R mutant. (**A,B,C**) Autoradiographs of gels separating phosphorylated peptides: peptide a (**A**), peptide b (**B**) and peptide c (**C**) respectively by wild-type EGF-R. Different concentrations of peptide substrates (0−3 mM final concentration) were added to equal aliquots of receptor immunoprecipitate and the phosphorylation reaction was started by addition of ATP (15 μM final concentration, 10 μCi [γ-^{32}P]ATP, 15 mM $MnCl_2$). After 2 min the reaction was stopped by addition of 3 × SDS sample buffer and heating to 95°C for 4 min. The reaction products were separated by SDS electrophoresis on a 5−15% polyacrylamide gel overlayed on a 20% acrylamide gel, allowing separate quantitation of receptor autophosphorylation and substrate phosphorylation. (**D,E,F**) Phosphorylation of peptide substrates by wild-type EGF-R. (**D**) Relative reaction-velocity (V/V_{max}) versus peptide concentration for peptides a, b and c. (**E**) Logarithmic plot of relative reaction velocity versus peptide concentration. (**F**) Lineweaver−Burk plot and regression parameters of data shown in (E). (**G,H,I**) Phosphorylation of peptide substrates by Y1068F EGF-R mutant. (**G**) Relative reaction-velocity (V/V_{max}) versus peptide concentration for peptides a, b and c. (**H**) Logarithmic plot of relative reaction velocity versus peptide concentration. (**I**) Lineweaver−Burk plot and regression parameters of data shown in (H).

the measured K_m values of Y1068F (70−100 μM), Y1173F (80−90 μM) and Y1173S (70−100 μM) were somewhat lower than those of the wild-type receptor (110−130 μM), and the observed differences could account for a 30−50% apparent increase in kinase activity. A decrease of activity through competitive inhibition as postulated by Bertics *et al.* (1988) would only be possible if the phenylalanine-containing site was a more potent inhibitor than the native tyrosine-containing site. This assumption is contradicted by inhibition constants determined for the tyrosine- and phenylalanine-containing peptides (Figure 5).

In conclusion, the effect of alteration of autophosphorylation sites on the kinase activity of the EGF-R do not exceed the effect expected for an internally competing substrate site. This small increase in kinase activity upon autophosphorylation is not directly involved in ligand activation. EGF stimulation increases the V_{max} of the protein tyrosine kinase, while autophosphorylation only affects the affinity of the substrate to the active site (K_m). It is not clear yet whether this modulation of activity is the primary function of EGF-R autophosphorylation. The physiological relevance of this modulation depends on the concentration of the substrates involved in growth regulation: if they are present at subsaturating concentration, modulation of the K_m of the receptor kinase could play a regulatory role, however, for substrates present at saturating concentration, small changes of the K_m would not have a significant effect. The fact that the mutated receptors are slightly more mitogenically active at low concentrations of EGF (Honegger *et al.*, 1988) suggests that the role of receptor autophosphorylation is to generate a stimulation threshold which has to be exceeded in order to induce a normal mitogenic response. The effects of replacing Y1068, Y1148 and Y1173 by phenylalanine are qualitatively and quantitatively similar, contradicting the special role of Y1173 postulated in earlier reports. The biological roles of the three sites seem to be very similar

and their influence on receptor activity appears to be exchangeable.

Materials and methods

Full details concerning the generation of the various constructs encoding for wild-type and mutant receptors, the transfection experiments and the cell lines expressing the receptor molecules are described in the accompanying manuscript (Honegger *et al.*, 1988).

Immunoprecipitation

Confluent cell layers were solubilized in 2 ml of lysis buffer per gram of cells. (Lysis buffer: 20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF.) The lysates were centrifuged in an Eppendorf centrifuge for 15 min and aliquots of the supernatant were stored at −70°C until used. Lysates containing approximately 10^7 cells were thawed, incubated for 10 min at 30°C in the presence or absence of 1 μg/ml EGF and immunoprecipitated with monoclonal antibody against EGF-R (mAb 108.1) bound to protein A−Sepharose. The washed immunoprecipitates were divided into 10 equal aliquots and used for the experiments.

Immunoblotting experiments

200 μl of SDS sample buffer were added to one aliquot of each receptor immunoprecipitate and heated to 95°C for 5 min. Aliquots of 100 μl, 50 μl, 25 μl, 12.5 μl, 6.25 μl and 3.125 μl were loaded into separate slots on a 7% SDS−polyacrylamide gel and electrophoretically separated overnight at a constant voltage of 60 V. The proteins in the gel were electrophoretically transferred to nitrocellulose for 60 min in an ABN Polyblot apparatus at a constant current of 400 mA. The blots were exposed to 5% dry milk powder in PBS for 60 min at room temperature, then incubated overnight at 4°C with anti-peptide antiserum against EGF-R, RK2 (Kris *et al.*, 1985) diluted 1:100 in milk/PBS, washed five times for 15 min at room temperature in milk/PBS, incubated for 2 h with 500 000 c.p.m./ml of [^{125}I]protein A in milk/PBS and again washed five times for 15 min with milk/PBS. The blots were rinsed in PBS, air-dried and analyzed by autoradiography. Radioactive bands were cut out and counted in a γ-counter.

Autophosphorylation

One aliquot of each receptor immunoprecipitate was resuspended in 1 ml of HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). Aliquots of 500 μl, 250 μl, 125 μl, 62.5 μl, 31.3 μl and 15.6 μl of the suspension were put in separate Eppendorf tubes, centrifuged for 5 min, and excess HNTG removed. 20 μl of HNTG and 10 μl of HNTG buffer containing 15 mM $MgCl_2$, 45 μM of ATP and 10 μCi of [γ-^{32}P]ATP were added to each vial and the reaction was allowed to proceed for 1 min at 4°C. The reaction was stopped by adding 15 μl of 3× SDS sample buffer and by heating to 95°C for 5 min. The samples were analyzed on a 7% SDS gel and, after autoradiography, the receptor bands were cut out and the amount of [^{32}P]phosphate incorporated determined by counting Cerenkov radiation in a β-counter.

Phosphopeptide maps

Radioactive bands of phosphorylated receptor were cut from gels and the SDS was eluted with 10% methanol. The gel slices were lyophilized and rehydrated with 50 μl of TPCK trypsin (Worthington) (1 mg/ml) in 50 mM ammonium bicarbonate pH 7.8. The gel slices were incubated overnight in 1 ml 50 mM ammonium bicarbonate at 37°C. The supernatant containing the eluted peptides was dried in a vacuum centrifuge. The phosphopeptides were dissolved in 0.1% TFA and analyzed by HPLC on a C_{18} column, using a 10-min isocratic wash with 0.1% TFA followed by a 30-min 0−30% linear gradient of acetonitrile in 0.1% TFA at a flow-rate of 1 ml/min. Fractions were collected every 30 s and counted in a β-counter.

Peptide phosphorylation

One aliquot of each receptor immunoprecipitate was resuspended in HNTG and divided into six tubes as described above. To each tube, 10 μl of HNTG, 10 μl of a 3 mM solution of synthetic peptide substrate (KGSTAENAEYLRV), and 10 μl of 15 mM $MnCl_2$, 45 μM ATP and 10 μCi of [γ-^{32}P]ATP in HNTG were added, incubated while shaking for 1 min at 4°C and stopped by adding 15 μl of 3 × SDS sample buffer and by heating to 95°C for 5 min. An SDS−polyacrylamide gel consisting of a bottom one-third of 20% polyacrylamide overlayed by a 5−15% gradient was used to separate the phosphorylated receptor from the phosphorylated peptide substrate from free ATP and phosphate. The gels were analyzed by autoradiography, and the receptor and peptide bands were excised and counted in a β-counter. One aliquot of each immunoprecipitate was incubated with 500 μl of 1 μM unlabeled ATP, 5 mM $MnCl_2$ and 200 mM Na-orthovanadate for 20 min at room temperature, then washed three times with HNTG and analyzed for autophosphorylation and peptide phosphorylation as described above.

Inhibition of autophosphorylation

10 μl of peptide solution (KGSTAENAEYLRV, KGSTAENAEFLRV or KGSTAENAEALRV) in HNTG was added to EGF-R immunoprecipitated from A431 cells (10 μl), mixed and incubated for 15−30 min on ice. 10 μl of 45 μM ATP, 10 μCi [γ-^{32}P]ATP and 15 mM $MnCl_2$ in HNTG were added and the reaction mixture was incubated for 2 min at 4°C under shaking. The reaction was stopped by adding 15 μl of 3 × SDS sample buffer and by heating to 95°C for 4 min. The samples were analyzed by SDS−PAGE and autoradiography.

Determination of K_m values for ATP and for peptide phosphorylation

Immunoprecipitates were divided equally into six tubes. Peptide phosphorylation was performed in the presence of 1 mM, 0.33 mM, 0.1 mM, 0.01 mM and 0.0033 mM of peptide respectively and with 10 μM ATP. Autophosphorylation was analyzed with 15 μM, 10 μM, 1.5 μM, 1 μM and 0.5 μM of ATP respectively, peptide phosphorylation in the presence of 0.2 mM peptide substrate and 15 μM, 10 μM, 5 μM, 1.5 μM, 1 μM and 0.05 μM ATP respectively. All the reactions were done in HNTG containing 5 mM $MnCl_2$ for 1 min at 4°C.

References

Baldwin,G.S., Knesel,J. and Monckton,J.M. (1983) *Nature*, **301**, 435−437.

Bertics,P.J. and Gill,G.N. (1985) *J. Biol. Chem.*, **260**, 14642−14647.

Bertics,P.J., Chen,W.S., Hubler,L., Lazar,C.S., Rosenfeld,M.G. and Gill,G.N. (1988) *J. Biol. Chem.*, **263**, 3610−3617.

Cartwright,C.A., Eckhardt,W., Simon,S. and Kaplan,P.L. (1987) *Cell*, **49**, 83−91.

Cassel,D., Pike,L.J., Grant,G.A., Krebs,E.G. and Glaser,L. (1983) *J. Biol. Chem.*, **258**, 2945−2950.

Downward,J., Parker,P. and Waterfield,M.D. (1984) *Nature*, **311**, 483−485.

Downward,J., Yarden,Y., Mayes,E., Scrace,G., Totty,N., Stockwell,P., Ullrich,A., Schlessinger,J. and Waterfield,M.D. (1984) *Nature*, **307**, 521−527.

Downward,J., Waterfield,M.D. and Parker,P.J. (1985) *J. Biol. Chem.*, **260**, 14538−14546.

Ellis,L., Clauser,E., Morgan,D.O., Edery,M., Roth,R.A. and Rutter,W.J. (1985) *Cell*, **45**, 721−732.

Erneux,C., Cohen,S. and Garbers,D.L. (1983) *J. Biol. Chem.*, **258**, 4137−4142.

Kmiecik,T.E. and Shalloway,D. (1987) *Cell*, **49**, 65−73.

Kris,R.M., Lax,I., Gullick,W., Waterfield,M.D., Ullrich,A., Fridkin,M. and Schlessinger,J. (1985) *Cell*, **40**, 619−625.

Livneh,E., Prywes,R., Kashles,O., Reiss,N., Sasson,I., Mory,Y., Ullrich, A. and Schlessinger,J. (1986) *J. Biol. Chem.*, **261**, 12490−12497.

Meckling-Hansen,K., Nelson,R., Branton,P. and Pawson,T. (1987) *EMBO J.*, **3**, 659−666.

Pike,L.J., Gallis,B., Casnellie,J.E., Bornstein,P. and Krebs,E.G. (1982) *Biochemistry*, **79**, 1443−1447.

Piwnica-Worms,H., Saunders,K.B., Roberts,T.M., Smith,A.E. and Cheng, S.H. (1987) *Cell*, **49**, 75−82.

Rosen,O.M., Herrera,R., Olowe,Y., Petruzzelli,L.M. and Cobb,M.H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3237−3240.

Stadtmauer,L. and Rosen,O.M. (1986) *J. Biol. Chem.*, **261**, 10000−10005.

Weber,W., Bertics,P.J. and Gill,G.N. (1984) *J. Biol. Chem.*, **259**, 14631−14636.

White,M.F., Haring,H.-U., Kasuga,M. and Kahn,C.R. (1984) *J. Biol. Chem.*, **259**, 255−264.

Yarden,Y. and Schlessinger,J. (1987) *Biochemistry*, **26**, 1434−1442.

Yarden,Y. and Ullrich,A. (1988) *Biochemistry*, **27**, 3113−3119.

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