Five enzymes of the glycolytic pathway serve as substrates for purified epidermal-growth-factor-receptor kinase

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Several enzymes of the glycolytic pathway are phosphorylated *in vitro* and *in vivo* by retroviral transforming protein kinases. These substrates include the enzymes phosphoglycerate mutase (PGM), enolase and lactate dehydrogenase (LDH). Here we show that purified EGF (epidermal growth factor)-receptor kinase phosphorylates the enzymes PGM and enolase and also the key regulatory enzymes of the glycolytic pathway, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in an EGF-dependent manner. Stoichiometry of phosphate incorporation into GAPDH (calculated from native M_r) is the highest, reaching ~ 1 . LDH and other enzymes of the glycolytic pathway are not phosphorylated by the purified EGF-receptor kinase. These enzymes are phosphorylated under native conditions, and the K_m values of EGF-receptor kinase for their phosphorylation are close to the physiological concentrations of these enzymes in the cell. EGF stimulates the reaction by 2–5-fold by increasing the V_{max} , without affecting the K_m of this process. Phosphorylation is rapid at 22 °C and at higher temperatures. However, unlike the self-phosphorylation of EGF-receptor, which occurs at 4 °C, the glycolytic enzymes are poorly phosphorylated at this temperature. Some enzymes, in particular enolase, increase the receptor K_m for ATP in the autophosphorylation process and thus may act as competitive inhibitors of EGF-receptor self-phosphorylation. On the basis of the K_m values of EGF receptor for the substrate enzymes and for ATP in the phosphorylation reaction, these enzymes may also be substrates *in vivo* for the EGF-receptor kinase.

INTRODUCTION

Several growth factors mediate their biological effects by activating membrane receptors which possess intrinsic tyrosine kinase activity [1–4]. This enzymic activity is also exhibited by transforming proteins belonging to the *src* gene family of certain retroviruses [5–8]. It is assumed [9–12] that by phosphorylation at tyrosine residues these growth-promoting proteins regulate target substrates which participate in biochemical pathways required for the initiation and support of cell proliferation. Therefore it is of great interest to identify and characterize the proteins which serve as substrates *in vivo* for tyrosine-specific protein kinases.

Although a variety of proteins and tyrosine-containing peptides were shown to serve as substrates *in vitro* for protein tyrosine kinases [13–18], only few proteins of defined function were identified as substrates *in vivo* for these kinases. Cooper *et al.* [19] have shown that the muscle isoenzymes of PGM and LDH, and the α isoenzyme of enolase, are among the few proteins that are phosphorylated on tyrosine residues in cells transformed by Rous sarcoma virus and other retroviruses. These enzymes are also phosphorylated at the same tyrosine residue when exposed to immunopurified retroviral protein kinases [20]. However, the stoichiometry of their phosphorylation was very low [20].

Elevated glycolytic activity is probably the most universal biochemical alteration detected in human and animal cancer cells. It is elevated in spontaneous human

and animal tumours [21], and in retroviral transformed cells [22–24]. Similarly, glycolytic activity is rapidly increased in quiescent normal cells treated with EGF [25–28], insulin [25] and serum [25]. The molecular alterations responsible for the above changes in glycolytic activity are not known. However, the activation of PFK by growth factors [25,28], serum [28] and retroviruses [29] was shown. To gain molecular insight into this problem, we have utilized purified EGF receptor to study whether phosphorylation of the above enzymes and other enzymes of the glycolytic pathway will occur.

We show here that several enzymes of the glycolytic pathway, including the key regulatory enzymes such as PFK and GAPDH, serve as substrates for purified EGF-receptor kinase.

EXPERIMENTAL

Materials

EGF was obtained from I.D.L., Jerusalem, Israel. [γ - 32 P]ATP (2000–3000 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Glycolytic enzymes (rabbit muscle) and Nonidet P40 were purchased from Sigma, St. Louis, MO, U.S.A. SDS was from BDH, Poole, Dorset, U.K. Cellulose-coated t.l.c. plates were from Merck, Darmstadt, Germany.

Preparation of A431 cell lysates

Confluent A431 cells, grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% (v/v) fetal-calf

Abbreviations used: EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PBS phosphate-buffered saline [10 mM-sodium phosphate (pH 7.2)/140 mM-NaCl]; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGM phosphoglycerate mutase.

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serum, 2 mM-glutamine and antibiotics, were washed three times in ice-cold PBS and solubilized in 1% Nonidet P40/50 mM-Hepes (pH 7.5)/1.5 mM-magnesium acetate/10% (v/v) glycerol/5 mM-EDTA/5 mM-EGTA, containing 10 µg of leupeptin/ml, 0.15 unit of aprotinin/ml and 1 mM-phenylmethanesulphonyl fluoride. Lysates were centrifuged at 12000 g for 15 min and the supernatants were stored at -80°C until used.

Preparation of A431 membranes

A431 cells were washed twice in PBS and once in 25 mM-Hepes (pH 7.5)/150 mM-NaCl/1 mM-EGTA (HNE buffer). Harvested cells were pelleted (600 g for 5 min) and incubated in 10 mM-Hepes (pH 7.5)/1 mM-EGTA/1.5 mM-magnesium acetate at 4°C for 10 min. After homogenization, nuclei were pelleted by centrifugation at 600 g for 20 min and the membranes by centrifugation at 40000 g for 60 min. The membrane pellet was resuspended in HNE buffer containing 10% glycerol and then centrifuged on a 35% (w/v)-sucrose cushion in HNE buffer at 18000 g for 2 h. The membrane interphase was collected, diluted 5-fold in 50 mM-Hepes buffer, pH 7.5, and pelleted at 40000 g for 60 min. Membranes were resuspended (at 3 mg/ml) in 20 mM-Hepes buffer, pH 7.5, containing proteinase inhibitors and stored at -80°C until used.

Immunoprecipitation of EGF receptor

Frozen A431-cell lysates were thawed, centrifuged (12000 g for 5 min), and the supernatant was treated with 2–10 µg of EGF/ml for 20 min at 4°C and then with Sepharose-bound monoclonal anti-EGF-receptor antibodies 29.1-IgG, as previously described [30]. After 2 h, the immobilized EGF receptor was washed three times with 1.0% Nonidet P40/2 M-KCl/50 mM-Hepes (pH 7.5)/10% glycerol and then three times with phosphorylation buffer (0.1% Nonidet P40/50 mM-Hepes, pH 7.5). No EGF was added after the immunoprecipitation of EGF receptor.

Phosphorylation of glycolytic enzymes

The phosphorylation reaction (30–50 µl) contained 0.2–1 µg of immunoprecipitated EGF receptor in 50 mM-Hepes (pH 7.5)/0.1% Nonidet P40/5 mM-MnCl₂/20 mM-2-mercaptoethanol (omitted when GAPDH was phosphorylated)/40 mM-KCl and varied concentrations of glycolytic enzymes and of [γ -³²P]ATP (1000–5000 c.p.m./pmol). The preparation of PFK obtained from Sigma contains 4 mM-ATP and 50 mM- β -glycerophosphate. Therefore it was washed three times in 90% (NH₄)₂SO₄. When A431 membranes were used as a source of EGF-receptor kinase, 0.01% saponin instead of Nonidet P40 and 100 µM-Na₃VO₄ were added. Reactions were terminated by addition of SDS/polyacrylamide-gel electrophoresis sample buffer [31].

Analysis of phosphorylated products

Proteins were separated by SDS/polyacrylamide-gel electrophoresis as described elsewhere [32] and stained for 30 min in 20% (v/v) acetic acid/20% (v/v) methanol/0.02% Coomassie Brilliant Blue. Gels were destained for 1 h in 20% (v/v) propan-2-ol, dried, and exposed to X-ray film with an intensifying screen. The bands of interest, located according to their autoradiography and staining positions, were excised and quantified by determining their Čerenkov radiation.

Phosphoamino acid analysis

Excised bands of phosphorylated protein were digested overnight at 37°C with trypsin in 0.05 M-NH₄HCO₃ (pH 7.9). Digested materials were freeze-dried and hydrolysed *in vacuo* in 6 M-HCl at 110°C for 2 h. Labelled phosphoamino acids were separated in the presence of unlabelled phosphoamino acid standards by two-dimensional electrophoresis on cellulose t.l.c. plates as described elsewhere [5]. Standard amino acids were stained with ninhydrin (0.25% in acetone) and the plates were exposed to X-ray film for several days at -80°C , by using an intensifier screen.

RESULTS

Purified EGF-receptor kinase phosphorylates several glycolytic enzymes

Enolase (rabbit muscle) is the best glycolytic-enzyme substrate *in vitro* of pp60^{src} [20]. Hence enolase was used to establish optimal conditions for phosphorylation by EGF-receptor kinase. Optimal phosphorylation of enolase (rabbit muscle isoenzyme) was obtained when the reaction mixture contained 0.2–1 µg of immunopurified EGF receptor in the presence of 20 mM-2-mercaptoethanol at 22°C or higher temperatures.

In addition to enolase, the following enzymes (rabbit muscle) were phosphorylated by the EGF-receptor kinase under similar conditions: PFK, GAPDH, PGK and PGM. The addition of EGF stimulated their phosphorylation by 2–5-fold (Fig. 1). EGF-enhanced substrate phosphorylation was retained when the substrates were individually phosphorylated or when present together in the reaction mixture, as may be the

Fig. 1. Phosphorylation of glycolytic enzymes by EGF-receptor kinase

EGF-receptor kinase, immunopurified in the absence (1) or presence (2) of EGF, was used for the phosphorylation of a mixture of glycolytic-enzyme substrates. The reaction was for 30 min at room temperature in the presence of 2 µM-ATP. Substrates were present at concentrations of $2 \times K_m$ values (except PGK, at $4 \times K_m$; see Table 1). Proteins were separated on a 10–20% polyacrylamide gradient. M_r markers (values on the right) were myosin, bovine serum albumin, ovalbumin, GAPDH and carbonic anhydrase. Other abbreviations: EGFR, EGF receptor; EN, enolase.

Fig. 2. Phosphoamino acid analysis of glycolytic-enzyme substrates

Phosphoamino acid analysis of the phosphorylated enzymes shown in Fig. 1 is described in the Experimental section. Phosphorylated glycolytic enzymes are (a) PFK, (b) GAPDH, (c) PGM and (d) enolase. The positions of unlabelled phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) standards are indicated.

case in living cells. None of the enzymes was phosphorylated when EGF receptor was omitted from the reaction mixture. Tyrosine is the only amino acid in these enzyme substrates that was phosphorylated by purified EGF-receptor kinase (Fig. 2). The chick muscle isoenzyme of enolase is not phosphorylated by EGF-receptor kinase. Similarly, pp60^{src} fails to phosphorylate this isoenzyme, probably because the target tyrosine residue of the mammalian enzyme is replaced by a histidine residue in chick muscle enolase [20].

Self-phosphorylation of EGF receptor occurs efficiently at both 4 °C and 22 °C. However, the phosphorylation of the glycolytic enzymes is drastically decreased at 4 °C (Fig. 3). 2-Mercaptoethanol is required for optimal phosphorylation of enolase, PFK, PGK and PGM. On the other hand, optimal phosphorylation of GAPDH was obtained in the absence of 2-mercaptoethanol. Since 2-mercaptoethanol does not have a significant effect on the self-phosphorylation of EGF receptor, it is probable that the action of the reducing agent is specific to the substrates rather than to EGF-receptor kinase. Moreover, it suggests that the susceptibility of these substrates to phosphorylation by EGF-receptor kinase may require free thiol groups in these enzymes in order to retain an appropriate substrate conformation.

All these enzymes were also phosphorylated by membrane preparations of A431 cells. However, the existence of phosphorylated polypeptides of similar M_r to PFK, PGK and enolase in A431-cell membranes

Fig. 3. Phosphorylation of GAPDH by EGF-receptor kinase as a function of temperature

GAPDH (4 μ M) was incubated at 4 °C (1) or at 22 °C (2) for 30 min with 6 μ g of A431-cell membranes and 5 μ g of EGF/ml, in the presence of 50 μ M-ATP, 100 μ M-sodium orthovanadate and 0.01% saponin. M_r markers (values on the right) for SDS/polyacrylamide-gel electrophoresis (7.5–18%-polyacrylamide gradient) are myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin and carbonic anhydrase. Abbreviation: EGFR, EGF receptor.

requires the utilization of purified EGF receptor for quantitative analysis.

LDH, a suitable substrate for transforming protein kinases *in vivo* [19] and *in vitro* [20,33], was not phosphorylated by EGF-receptor kinase. Similarly, triose-phosphate isomerase and pyruvate kinase are not phosphorylated by EGF-receptor kinase. Aldolase, phosphoglucosomerase, and phosphoglucosomutase are marginally phosphorylated by EGF-receptor kinase under the present conditions.

Time course of phosphorylation of glycolytic enzymes

A similar time course was obtained for the phosphorylation of the substrate enzymes. Fig. 4 shows the phosphorylation of substrate enzymes by EGF-receptor kinase, immunoprecipitated in the absence or presence of EGF, as a function of time. By 20 s, phosphate incorporation into glycolytic enzymes was detected. The rate of the reaction is linear for approx. 15 min and levels off to a plateau after 30 min. The stoichiometry of GAPDH phosphorylation is most prominent compared with phosphorylation of the other enzymes, including the 20 s time point. The stoichiometry of phosphate incorporation into GAPDH (calculated from native M_r) is almost 1 (0.8 mol of phosphate/mol of GAPDH). The extent of phosphate incorporation into enolase (0.2 mol/mol), PGM (0.05 mol/mol), PFK (0.2 mol/mol) and PGK (0.02 mol/mol) is much higher than the extent of phosphorylation previously reported [20] for PGM and enolase by other protein-tyrosine kinases. The marginal phosphorylation of glycolytic enzymes observed after 30 min reaction is probably due to the thermal deactivation of the EGF-receptor kinase. We studied the thermal stability of EGF-receptor kinase. After 30 min incubation of immunopurified EGF receptor at room temperature, in the presence of ATP (Fig. 5) as well as in its absence (results not shown), at most 20% of the

Fig. 4. Time course of phosphorylation of glycolytic enzymes

PFK (a), GAPDH (b), PGM (c) and enolase (d), at concentration of $2 \times K_m$ values (Table 1), were phosphorylated as described in the Experimental section by EGF-receptor kinase immunopurified in the absence (●) or presence (○) of EGF. The determination of the radioactivity of phosphorylated proteins is described in the Experimental section.

original kinase activity is observed. At 37 °C, already after 5 min the EGF-receptor kinase became inactive.

Kinetic parameters of phosphorylation of glycolytic enzymes by EGF-receptor kinase

The apparent K_m values and the maximum velocity of the phosphorylation reaction of glycolytic enzymes were

obtained from double-reciprocal plots of reaction rates as a function of substrate concentration (calculated from the native M_r) and are summarized in Table 1. Both PFK and GAPDH exhibited high affinities for EGF-receptor kinase (apparent K_m values of $0.09 \mu\text{M}$ and $0.48 \mu\text{M}$ respectively). The K_m values for the other glycolytic enzymes are in the range $1\text{--}2 \mu\text{M}$. EGF does not affect the K_m values of the reaction; it does, however, increase the V_{max} of this process by 2–5-fold. The best substrate for EGF-receptor kinase *in vitro* is GAPDH, which accepted the highest amount of phosphate and also showed 5-fold stimulation of phosphorylation in the presence of EGF. The phosphorylation of the other substrates varied according to their K_m and V_{max} values. Enolase increased the K_m of EGF receptor for ATP in the autophosphorylation reaction, thus diminishing self-phosphorylation of EGF receptor by 70% of its original value (Fig. 5). However, the kinase activity of EGF receptor was not affected, since enolase itself was phosphorylated under these conditions (Fig. 5).

We measured the rate of phosphorylation of glycolytic enzymes as a function of ATP concentration. The apparent K_m value for ATP, determined from a Lineweaver–Burk plot, was the lowest for GAPDH (Fig. 6 and Table 2) and highest for PGM (Table 2). Nevertheless, the K_m values of EGF-receptor kinase for ATP for the phosphorylation of substrate enzymes are below the concentration of ATP in living cells.

Similar to the findings that EGF does not alter the K_m values of EGF receptor for glycolytic enzymes, EGF does not affect the K_m values for ATP, and only the kinase V_{max} values were affected by EGF. Unexpectedly, at high ATP concentration, the basal phosphorylation of PGM was almost equal to the phosphorylation by EGF-stimulated kinase (see Table 2). The EGF receptor K_m for ATP for the autophosphorylation reaction is in the range $4\text{--}8 \mu\text{M}$. This value is not altered in the presence of PFK, GAPDH and PGM. However, enolase ($2 \mu\text{M}$) increased the apparent K_m value for ATP in the

Fig. 5. Phosphorylation of increasing concentrations of enolase

Lanes 1 and 7 contain only EGF receptor (EGFR). Enolase (EN) was present at increasing concentrations: $0.15 \mu\text{M}$ (lanes 2 and 8), $0.45 \mu\text{M}$ (lanes 3 and 9), $1.5 \mu\text{M}$ (lanes 4 and 10), $4.5 \mu\text{M}$ (lanes 5 and 11) and $8 \mu\text{M}$ (lanes 6 and 12). In lanes 1–6, ATP ($50 \mu\text{M}$; 2000 c.p.m./pmol) was added after the addition of enolase and EGF receptor. The reaction mixture was then incubated for 30 min at room temperature. In lanes 7–12, immunopurified receptor was first phosphorylated for 30 min at room temperature and then enolase was added for an additional period of 30 min. M_r markers (values on the right) are myosin, β -galactosidase, phosphorylase b, bovine serum albumin, ovalbumin and carbonic anhydrase.

Table 1. Kinetic parameters for the phosphorylation of glycolytic enzymes by EGF-receptor kinase

Glycolytic enzymes were phosphorylated by EGF receptor immunoprecipitated in the absence and presence of EGF (5 $\mu\text{g/ml}$) as described in the legend to Fig. 5 and the Experimental section. The K_m and V_{max} , calculated from the native M_r of substrates, were derived from Lineweaver–Burk plots by using a line-fitting routine.

Substrate	Without EGF		With EGF	
	Apparent K_m (μM)	Apparent V_{max} (pmol/min)	Apparent K_m (μM)	Apparent V_{max} (pmol/min)
PFK	0.095	0.08	0.09	0.22
GAPDH	0.52	0.30	0.48	1.18
PGK	1.80	0.09	2.00	0.22
PGM	1.60	0.06	1.56	0.13
Enolase	0.96	0.15	1.00	0.4

Fig. 6. Double-reciprocal plot of GAPDH phosphorylation by EGF-receptor kinase as a function of ATP concentration

GAPDH was phosphorylated for 15 min by EGF-receptor kinase immunopurified in the absence (●; lanes 1–5) or presence (○; lanes 6–10) of EGF (5 $\mu\text{g/ml}$) with various concentrations of ATP (5 μM , lanes 1 and 6; 10 μM , lanes 2 and 7; 50 μM , lanes 3 and 8; 100 μM , lanes 4 and 9; 250 μM , lanes 5 and 10). Lineweaver–Burk plots were drawn after determination of phosphate incorporation into the polypeptide bands separated by SDS/polyacrylamide-gel electrophoresis (see the insert) as described in the Experimental section. M_r markers (values on left of the gels) were phosphorylase *b*, bovine serum albumin, ovalbumin and carbonic anhydrase. Abbreviation: EGFR, EGF receptor.

autophosphorylation reaction to 28 μM . This result is consistent with the decreased autophosphorylation of EGF receptor in the presence of high concentrations of enolase (Fig. 5).

DISCUSSION

Serum and growth factors enhance the glycolytic activity in normal mitogenically responsive cells [25–28].

Moreover, this metabolic pathway appears to be constitutively activated in many human and animal tumour cells. Hence, we decided to examine the possibility that purified EGF-receptor kinase may directly phosphorylate glycolytic enzymes, which may result in modulating their activity. We also decided to explore the capacity of EGF to enhance the phosphorylation of these enzymes in living cells.

The major conclusions from the present study are as

Table 2. Kinetic parameters for phosphorylation of glycolytic enzymes by EGF-receptor kinase as a function of ATP concentration

Glycolytic enzymes at concentrations of $2 \times K_m$ values (except PGK, at $4 \times K_m$; see Table 1) were phosphorylated by EGF receptor immunopurified in the absence or presence of EGF (5 $\mu\text{g/ml}$) as described in the Experimental section, and in the presence of various concentrations (2–1000 μM) of [γ - ^{32}P]ATP. K_m and V_{max} values were estimated by double-reciprocal plots as shown in Fig. 6, by using a line-fitting routine.

Substrate	Without EGF		With EGF	
	Apparent K_m (μM)	Apparent V_{max} (pmol/min)	Apparent K_m (μM)	Apparent V_{max} (pmol/min)
PFK	125	0.052	125	0.15
GAPDH	12	0.12	12	0.60
PGK	263	0.045	250	0.09
PGM*	500	0.26	500	0.34
Enolase	165	0.12	145	0.30

* For PGM, the double-reciprocal plot of rate versus ATP concentration is non-linear (curving down at high ATP concentrations). Therefore only the points at high (50–1000 μM) ATP concentrations (which provide a linear plot) were used.

follows. The purified EGF-receptor kinase phosphorylates several glycolytic enzymes, including key regulatory enzymes of the glycolytic pathway. The enzymes are phosphorylated under native conditions, showing affinities which are close to their physiological concentration in most cells. Phosphorylation is rapid at 22 °C and at higher temperatures. EGF acts by increasing the V_{max} of this process. Unlike the self-phosphorylation of EGF receptor, the glycolytic enzymes are marginally phosphorylated at 4 °C. The K_m values measured for the phosphorylation of these enzymes are among the lowest values measured for known substrates to protein tyrosine kinases [15–18,34]. In addition, the K_m values for ATP for the phosphorylation of these enzymes are in the range of physiological ATP concentrations. It is noteworthy that at high ATP concentrations the phosphorylation of PGM by EGF-stimulated kinase is marginally higher than the phosphorylation obtained with untreated receptor. It seems that, in the presence of high concentrations of ATP, PGM is efficiently phosphorylated even in the absence of the growth factor. The highest stoichiometry of phosphate incorporation (~ 1) was obtained for GAPDH, whereas PFK showed the highest affinity for EGF-receptor kinase. Nevertheless, more efforts to obtain quantitatively phosphorylated enzymes are required before it will be possible to determine the effect of tyrosine phosphorylation on enzyme properties.

It was previously shown that pp60^{src} and other transforming proteins belonging to the *src* gene family are able to phosphorylate some of the glycolytic enzymes both *in vivo* [19] and *in vitro* [20,33]. In spite of the lack of detailed characterization of glycolytic-enzyme phosphorylation by viral transforming protein kinases [20,33], it is clear that there are several differences between EGF receptor and pp60^{v-src} concerning their substrate specificity and extent of phosphorylation. Enolase and PGM, which serve as substrates *in vivo* [19] and *in vitro* [20,33] for retroviral protein kinases, are also phosphorylated by EGF-receptor kinase. Similar K_m values were obtained for the phosphorylation of enolase by EGF-receptor kinase and by retroviral protein kinases [20]. However, the extent of phosphorylation of PGM

and enolase by EGF-receptor kinase is much greater. LDH is phosphorylated both *in vivo* and *in vitro* by retroviral protein kinases, but is not phosphorylated by EGF-receptor kinase. On the other hand, PGK is phosphorylated by EGF-receptor kinase, but not by the retroviral protein tyrosine kinase [33]. PFK, which is a key regulatory enzyme of the glycolytic pathway, exhibits high affinity for EGF-receptor kinase, but was only marginally phosphorylated by retroviral transforming proteins [33]. The reason for and significance of the differences in extent and specificity of phosphorylation of glycolytic enzymes by EGF-receptor kinase and retroviral protein tyrosine kinases have yet to be established.

We do not know yet whether these enzymes are directly phosphorylated by an EGF-dependent process in intact cells. The K_m values of EGF receptor for the substrate enzymes and for ATP indicate that enolase, GAPDH and PFK may be phosphorylated by EGF receptor in living cells. In preliminary experiments, we found that in A431 cells treated for 1 h with EGF or the tumour promoter phorbol ester (TPA) the phosphorylation of some of these enzymes is increased (N. Reiss, H. Kanety & J. Schlessinger, unpublished work). The phosphorylation was on serine and threonine residues. Hence, it is possible that the Ca^{2+} and phospholipid-dependent protein kinase-C or other serine/threonine kinases may phosphorylate these enzymes in living cells. More experiments are required to establish whether EGF will induce tyrosine phosphorylation of glycolytic enzymes in living cells. Nevertheless, although glycolytic enzymes are phosphorylated on tyrosine in retrovirus-transformed cells [19], it is not yet clear whether tyrosine phosphorylation of the glycolytic enzymes plays any role in the regulation of their activity.

The effect of the glycolytic enzymes on the self-phosphorylation of EGF-receptor deserves comment. Enolase may act as a competitive inhibitor of this process. Inhibition of self-phosphorylation of EGF receptor by millimolar concentrations of peptide substrates was reported [35–37]. However, no documentation of the effect of these substrates on the receptor K_m for ATP in the autophosphorylation process was provided. Our data suggest that, at micromolar concentrations,

enolase decreases the receptor affinity for ATP in the self-phosphorylation reaction.

In summary, we have shown that the purified EGF-receptor kinase phosphorylates several glycolytic enzymes. It remains to be seen whether this or similar processes occur in living cells and whether the phosphorylation state of these enzymes will regulate their activity.

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REFERENCES

1. Ushiro, H. & Cohen, S. (1980) *J. Biol. Chem.* **255**, 8363-8365
2. Buhrow, S. A., Cohen, S. & Stavros, J. V. (1982) *J. Biol. Chem.* **257**, 4019-4022
3. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. (1983) *J. Biol. Chem.* **258**, 10973-10980
4. Roth, R. A. & Cassell, D. J. (1983) *Science* **219**, 299-301
5. Sefton, B. M., Hunter, T., Beemon, K. & Eckhart, W. (1980) *Cell* **20**, 807-816
6. Erikson, E. & Erikson, R. L. (1980) *Cell* **21**, 829-836
7. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311-1315
8. Cooper, J. A. & Hunter, T. (1981) *Mol. Cell. Biol.* **1**, 165-178
9. Karess, R. E., Hayward, W. S. & Hanafusa, H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3154-3158
10. Witte, O. N., Dasgupta, A. & Baltimore, D. (1980) *Nature (London)* **283**, 826-831
11. Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P. & Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1443-1447
12. White, M. F., Haring, H.-U., Kasuga, M. & Kahn, C. R. (1984) *J. Biol. Chem.* **259**, 255-264
13. Hunter, T. (1982) *J. Biol. Chem.* **257**, 4843-4848
14. Radke, K., Gilmore, T. & Martin, S. G. (1980) *Cell* **21**, 821-828
15. Kadowaki, T., Fujita-Yamaguchi, Y., Nishida, E., Takaku, F., Akiyama, T., Kathuria, S., Akanuma, Y. & Kasuga, M. (1985) *J. Biol. Chem.* **260**, 4016-4020
16. Pike, L. J., Kunzel, E. A., Casnellie, J. E. & Krebs, E. G. (1984) *J. Biol. Chem.* **259**, 9913-9921
17. Baldwin, G. S., Burgess, A. W. & Kemp, B. E. (1982) *Biochem. Biophys. Res. Commun.* **109**, 656-663
18. Ghosh-Dastidar, P., Coty, W. A., Griest, R. E., Woo, D. D. L. & Fox, F. C. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1654-1658
19. Cooper, J. A., Reiss, N. A., Schwartz, R. J. & Hunter, T. (1983) *Nature (London)* **302**, 218-223
20. Cooper, J. A., Esch, F. S., Taylor, S. S. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 7835-7841
21. Warburg, O. (1926) *Über den Stoffwechsel der Tumoren*, Springer Verlag, Berlin
22. Hatanaka, M. & Hanafusa, H. (1980) *Virology* **41**, 647-652
23. Singh, V. N., Singh, M., August, J. T. & Horecker, B. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4129-4132
24. Bissel, M. J., White, R. C., Hatie, C. & Bassham, J. A. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2951-2955
25. Diamond, I., Legg, A., Schneider, J. A. & Rozengurt, E. (1978) *J. Biol. Chem.* **253**, 866-871
26. Boerner, P., Resnick, R. J. & Racker, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1350-1353
27. Inman, W. H. & Colowick, S. P. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1346-1349
28. Schneider, J. A., Diamond, I. & Rozengurt, E. (1978) *J. Biol. Chem.* **253**, 872-877
29. Fodge, D. W. & Rubin, H. (1973) *Nature (London)* **246**, 181-183
30. Yarden, Y., Harari, I. & Schlessinger, J. (1985) *J. Biol. Chem.* **260**, 315-319
31. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
32. Reiss, N. A. & Kaye, A. M. (1981) *J. Biol. Chem.* **256**, 5741-5749
33. Coussens, P. M., Cooper, J. A., Hunter, T. & Shalloway, D. (1985) *Mol. Cell. Biol.* **5**, 2753-2763
34. Braun, S., Raymond, W. E. & Racker, E. (1984) *J. Biol. Chem.* **259**, 2051-2054
35. Cassel, D., Pike, L. J., Grant, G. A., Krebs, E. G. & Glaser, L. (1983) *J. Biol. Chem.* **258**, 2945-2950
36. Bertics, P. J. & Gill, G. N. (1985) *J. Biol. Chem.* **260**, 14642-14647
37. Downward, J., Waterfield, M. D. & Parker, P. J. (1985) *J. Biol. Chem.* **260**, 14538-14546

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