Volume 250, number 2, 469-473 FEB 07318 July 1989

The 18 kDa cytosolic acid phosphatase from bovine liver has phosphotyrosine phosphatase activity on the autophosphorylated epidermal growth factor receptor

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Received 1 May 1989

In this paper we demonstrate that the cytosolic low-M_r acid phosphatase purified from bovine liver has phosphotyrosine protein phosphatase acitivity on ^{32}P-autophosphorylated epidermal growth factor (EGF) receptor. This activity was significantly inhibited by orthovanadate and *p*-hydroxymercuribenzoate; the latter result indicates that free sulfhydryl groups are required for phosphotyrosine phosphatase activity. The enzyme was active in a broad pH range, with maximum activity between pH 5.5 and 7.5. The apparent K_m for ^{32}P-EGF receptor dephosphorylation was 4 nM. The enzyme appeared to be specific for phosphotyrosine in that it dephosphorylated the autophosphorylated EGF receptor and L-phosphotyrosine, but not ^{32}P-Ser-casein, L-phosphoserine or L-phosphothreonine. These data suggest that the cytosolic low-M_r acid phosphatase might play a regulatory role in EGF receptor-dependent transmembrane signalling.

Acid phosphatase; Epidermal growth factor receptor; Phosphotyrosine

1. INTRODUCTION

The epidermal growth factor (EGF) receptor is a 170 kDa transmembrane glycoprotein with intrinsic tyrosine-specific protein kinase activity. Activation of the EGF receptor induces a complex series of events that include the phosphorylation of several protein substrates, receptor autophosphorylation, stimulation of inositol lipid turnover, increase of cytosolic pH and Ca^{2+} concentration, and mitogenesis (reviews see [1]). Receptor-kinase activation appears to be required for signal transduction, since tyrosine kinase-deficient mutant receptors fail to induce any biomedical or biological response to EGF [2]. EGF receptor autophosphorylation itself depends on the sustained presence of EGF; once removed, rapid receptor dephosphorylation generally follows [2]. This sequence conforms to a scheme of coordinated kinase and phosphatase activities already established as a general mechanism of enzyme regulation [3]. Thus, it is likely that phosphotyrosyl-specific protein phosphatases also play a role in growth factor-dependent signal pathways.

Acid phosphatases (EC 3.1.3.2) have been described in a variety of tissues in vertebrates; these enzymes differ from each other in molecular mass, cellular localization, and sensitivity to different effectors [4]. Three or four classes of different molecular mass enzymes have been described so far [4,5], and there is evidence that some of these enzymes might be specific for phosphotyrosyl residues [6].

We have recently determined the complete primary structure of the low-M_r cytosolic acid phosphatase purified from bovine liver [7]. Here, we characterized the activity of this enzyme on the

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

human EGF receptor overexpressed in NR6 fibroblasts, an NIH/3T3 mutant cell line devoid of EGF receptors [8]. Our results demonstrate that the low-M_r acid phosphatase dephosphorylates this biologically relevant substrate, suggesting that it may be involved in EGF signalling.

2. MATERIALS AND METHODS

Dephosphorylated casein, the catalytic subunit of cAMP-dependent protein kinase, L-phosphoserine, L-phosphothreonine and L-phosphotyrosine were obtained from Sigma; *p*-nitrophenyl phosphate and *p*-hydroxymercuribenzoate from Serva; Orthovanadate from Aldrich; and [γ-^{32}P]ATP (5000 Ci/mmol) from Amersham. All other reagents were of the highest qualitiy available.

2.1. *Preparation of the EGF receptor*

EGF receptor was partially purified from NR6 fibroblasts overexpressing the transfected human EGF receptor [8] by wheat germ-agglutinin chromatography as described [9]. In these preparations, EGF receptor autophosphorylation could be stimulated by 0.66 μM EGF or 5 mM $MnCl_2$ as described previously [9], without detectable differences in stoichiometry or tyrosine specificity. EGF receptor constituted >95% of the phosphotyrosyl-protein content, as determined by SDS-PAGE autoradiography of [γ-^{32}P]ATP-labeled samples (not shown).

2.2. *Preparation of the low-M_r acid phosphatase*

The enzyme was purified as in [4]. The preparation consisted of pure protein with specific activity of about 110 U/mg protein at 37°C, using *p*-nitrophenyl phosphate as substrate.

2.3. *Autophosphorylation of EGF receptor*

The EGF receptor preparation was mixed in a total volume of 0.5 ml with 0.02 M Hepes buffer (pH 7.4), 5 mM $MnCl_2$, 5 μM [γ-^{32}P]ATP (spec. act. 39 μCi/nmol) and 0.04% Triton X-100 (final concentrations). The reaction was started by adding the receptor preparation and the mixture was incubated for 25 min at room temperature. The phosphorylated receptor was then purified by gel filtration on a Bio-Gel P6 column (1.5 × 5 cm) equilibrated with 0.04% Triton X-100 and the void volume was exhaustively dialyzed vs 0.04% Triton X-100 solution. Phosphorylation of the EGF receptor was assessed by SDS-PAGE [10], followed by contact radioautography.

2.4. *Acid phosphatase assay*

4 mM *p*-nitrophenyl phosphate in 0.1 M acetate buffer (pH 5.5) containing EDTA (final volume, 1 ml) was used to determine phosphatase activity. Incubation was performed at 37°C for 1–5 min. The reaction was terminated by addition of 4 ml of 0.1 M KOH and released *p*-nitrophenol was measured spectrophotometrically at 400 nm.

2.5 *Phosphoamino acid phosphatase activity*

Tyrosine phosphate phosphatase activity was determined on L-tyrosine phosphate according to Apostol et al. [11]. Dephosphorylation of L-phosphoserine and L-phosphothreonine was measured by the method of Baginski [12].

2.6. *Dephosphorylation of ^{32}P-EGF receptor and ^{32}P-Ser-casein*

The dephosphorylation activity of the low-M_r acid phosphatase was monitored by measuring the release of $^{32}P_i$. Approx. 2.3 nM (as phosphate groups) ^{32}P-EGF receptor in 40 μl reaction mixture containing 0.03 M buffer (β,β-dimethylglutarate in the range pH 3.2–7.6; Hepes, pH 7.4), and 0.04% Triton X-100, was incubated in the presence of acid phosphatase at 37°C for different periods of time. The reaction was stopped by the addition of 12 μl of 0.6% bovine serum albumin followed by 120 μl of 20% trichloroacetic acid. After centrifugation at 12 000 × g, $^{32}P_i$ was measured by liquid scintillation counting. In the experiments designed to determine pH optimum and K_m, the amount of acid phosphatase and incubation period were adjusted in order to achieve less than 20% dephosphorylation of the receptor. Casein was phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP as described by Chernoff et al. [13]. ^{32}P-Ser-casein dephosphorylation was measured essentially as described for the EGF receptor, but using approx. 2.5 nM (as phosphate groups) ^{32}P-Ser-casein as substrate.

3. RESULTS AND DISCUSSION

The effect of the low M_r acid phosphatase on the autophosphorylated EGF receptor is shown in fig.1. The [^{32}P]phosphorylated receptor migrated as a single band of about 170 kDa on SDS-PAGE (lane −). Addition of the phosphatase caused dramatic dephosphorylation of this band as shown in fig.1 (lane +). The kinetics of dephosphorylation and the sensitivity to different inhibitors are shown in fig.2. The kinetics were linear for up to 60 min, and increasing concentrations of orthovanadate effectively inhibited dephosphorylation. *p*-Hydroxymercuribenzoate (200 μM) was also inhibitory on the phosphatase activity; this latter result is in agreement with the observation that cysteine residues are part of the active site of the enzyme [7]. The apparent pH optimum for EGF receptor dephosphorylation was between pH 5.5 and 7.5, as shown in fig.3. The K_m for dephosphorylation of the autophosphorylated receptor was 4 nM, as calculated from the experiment shown in fig.4. To determine whether this effect was specific for phosphotyrosyl residues, we measured phosphatase activity on ^{32}P-Ser-casein. Fig.5 shows that the low-M_r acid phosphatase from bovine liver did not dephosphorylate ^{32}P-Ser-casein. Furthermore, we analyzed phosphoamino

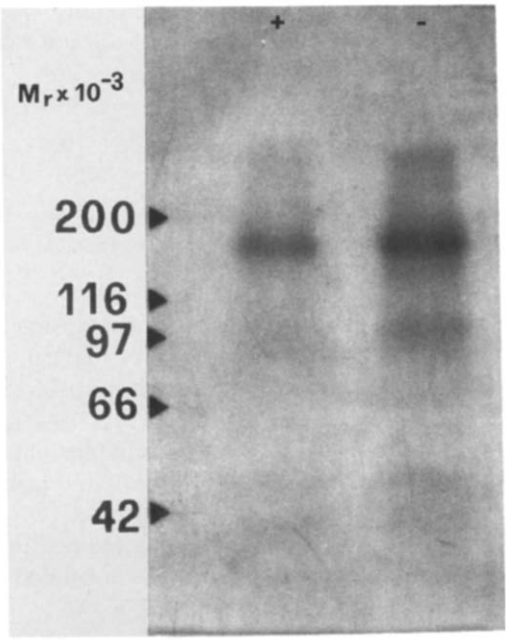

Fig.1. Dephosphosphorylation of the EGF receptor by the low-M_r acid phosphatase. The ^{32}P-autophosphorylated EGF receptor, containing about 30 000 cpm $^{32}P_i$, was incubated with (+) or without (−) the low M_r acid phosphatase in 40 mM Hepes buffer, pH 7.4, at 37°C for 90 min. The reaction was terminated by adding Laemmli sample buffer containing 100 mM DTT. The samples were analyzed by SDS-PAGE (7% gel). A representative autoradiogram is shown.

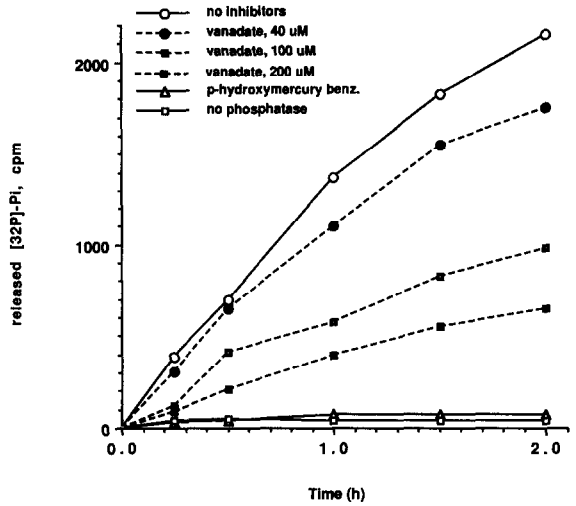

Fig.2. Time course of EGF receptor dephosphorylation and sensitivity to inhibitors. These experiments were performed using ^{32}P-EGF receptor (2.4 nM as phosphate groups), 1 μg low-M_r acid phosphatase in 0.04 Hepes buffer (40 μl, total volume), pH 7.4, different inhibitors at the indicated concentrations, and control containing ^{32}P-EGF receptor, but no acid phosphatase. This experiment was performed three times with identical results. Data are expressed as cpm of released $^{32}P_i$.

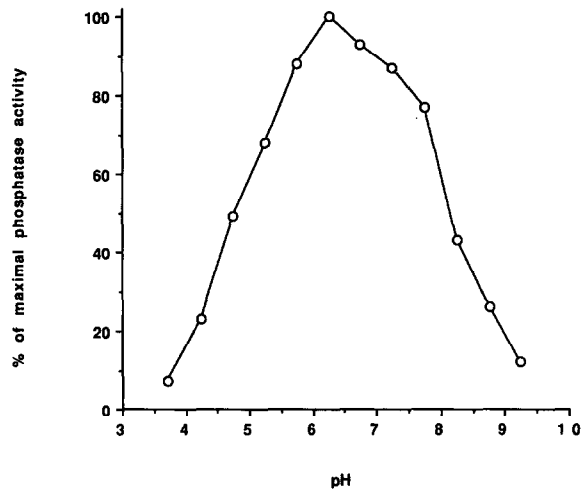

Fig.3. pH optimum for EGF receptor dephosphorylation. Experiments were performed using 0.04 M β,β-dimethylglutarate buffer (pH 3.7–7.6) and 0.04 M Tris-HCl buffer (pH 8.1–9.1). This experiment was performed three times with identical results. Data are expressed as % of maximal activity.

acid dephosphorylation (table 1) and observed that L-phosphotyrosine was the only phosphoamino acid that was dephosphorylated by the enzyme. The relative activity on L-phosphotyrosine, as compared to *p*-nitrophenyl phosphate, was about 44%. Taken together, these results show that the low-M_r acid phosphatase from bovine liver specifically dephosphorylates phosphotyrosine but not phosphoserine or phosphothreonine.

Several growth factor receptors and oncogene products have tyrosine-specific protein kinase activity [14], suggesting that tyrosine phosphorylation of specific protein substrates is crucial for cell growth. Since the phosphorylated state of protein substrates results from the balance of protein kinase and protein phosphatase activities, phosphotyrosyl-specific protein phosphatases are also likely to play an important role in the control of cell proliferation. Indirect evidence for this role

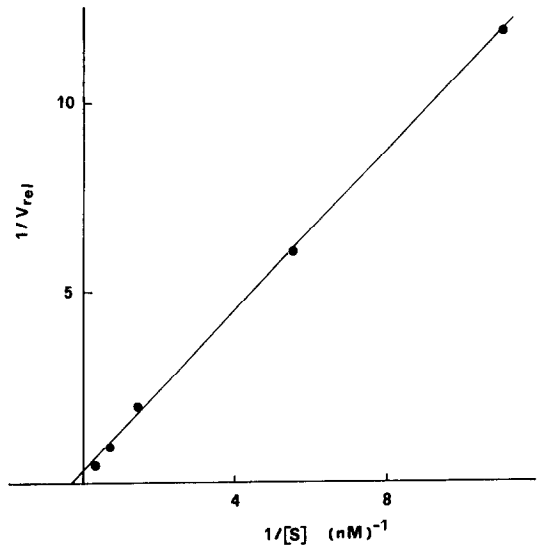

Fig.4. Lineweaver-Burk plot for phosphotyrosyl protein phosphatase activity of the low-M_r acid phosphatase using the autophosphorylated EGF receptor as substrate. The apparent K_m calculated from this plot is 4 nM. This experiment is representative of three others that gave identical results.

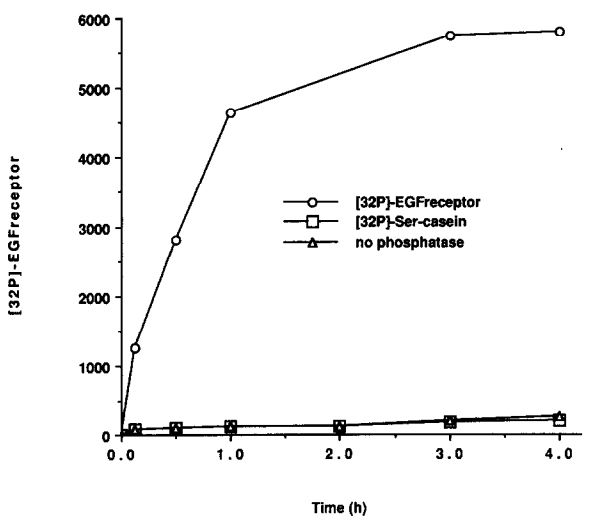

Fig.5. Effect of the low-M_r acid phosphatase on the ^{32}P-autophosphorylated EGF receptor and on ^{32}P-Ser-casein. Experiments were performed using ^{32}P-EGF receptor (2.3 nM as phosphate groups), 2 μg acid phosphatase in 0.04 M Hepes buffer, pH 7.4, ^{32}P-Ser-casein (2.5 nM as phosphate groups), and control containing ^{32}P-EGF receptor, but no acid phosphatase. Results, expressed as cpm of released $^{32}P_i$, are from a representative experiment, one out of three that gave identical results.

Table 1
Phosphoamino acid specificity of the low-M_r acid phosphatase

Substrate	Relative activity (%)
p-Nitrophenyl phosphate	100
L-Phosphotyrosine	44
L-Phosphoserine	0.2
L-Phosphothreonine	0.01

has been obtained from experiments using orthovanadate, a phosphatase inhibitor that causes a dramatic increase in cellular phosphotyrosine content and cell transformation [15].

Our data show that the low-M_r acid phosphatase dephosphorylated the activated EGF receptor in vitro. The specificity of phosphatase activity and its inhibition by orthovanadate raise the possibility of a regulatory role for this enzyme in EGF signal transduction.

Acknowledgements: This work was supported by grants from the Italian Ministero della Pubblica Istruzione. M.R. was supported in part by a grant from the Italian Association for Cancer Research (AIRC). The authors thank Dr S.A. Aaronson for critical reading of this manuscript and helpful discussion.

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