Alteration of the kinetic properties of the epidermal growth factor receptor tyrosine kinase by basic proteins

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Previous studies have shown that lysine- and arginine-rich proteins can enhance the activity of tyrosine and serine/threonine protein kinases. However, the kinetics and mechanism of this activation are not fully understood. Therefore we investigated the ability of poly(amino acids) and the arginine-rich protein, protamine, to alter the kinetic properties of epidermal growth factor (EGF) receptor protein-tyrosine kinase activity using immunoaffinity-purified receptor isolated from human epidermoid carcinoma (A431) cells. Poly(L-lysine), poly(L-arginine) and protamine stimulated EGF receptor kinase activity by 3–5-fold at non-saturating doses of ATP and peptide substrate, while poly(L-glutamate) had no effect. Initial kinetic studies demonstrated an increase in the maximum velocity and a decrease in the apparent K_m for the peptide substrate angiotensin II in the presence of the basic effectors. Further analysis of the kinetic mechanism by product inhibition revealed that protamine altered the pattern of ADP inhibition towards the peptide substrate but not towards ATP. The change was indicative of the receptor's ability to form an enzyme–angiotensin II–ADP ternary complex in the presence of protamine but not in its absence. In addition, the basic effectors had a substantially decreased influence on the kinase activity of a C-terminally truncated form of the EGF receptor. Thus the changes in kinase activity may be partially mediated by the C-terminal region of the receptor, which contains the sites of receptor self-phosphorylation. These results suggest that the basic domains of proteins can interact with the EGF receptor to induce changes in its kinetic properties, especially with regard to reactant recognition and binding.

INTRODUCTION

The epidermal growth factor (EGF) receptor is a 170 kDa transmembrane glycoprotein containing an extracellular EGF-binding domain and an intracellular domain that exhibits ligand-stimulable protein-tyrosine kinase activity (Hunter & Cooper, 1985; Carpenter, 1987; Yarden & Ullrich, 1988; Carpenter & Wahl, 1990; Ullrich & Schlessinger, 1990). In many cell types receptor activation leads to increased tyrosine and serine/threonine phosphorylation of intracellular proteins (Hunter & Cooper, 1981), elevation in intracellular free Ca^{2+} levels and Na^+/H^+ exchange, increased gene transcription, and ultimately DNA synthesis and cell division (Carpenter & Cohen, 1979). The EGF receptor phosphorylates itself in a rapid, reversible reaction (Hubler *et al.*, 1989), and four major sites of self-phosphorylation have been identified in the extreme C-terminus of the receptor (Downward *et al.*, 1984; Margolis *et al.*, 1989). Our earlier kinetic studies have indicated that this region can act as a competitive constraint on exogenous substrate phosphorylation (Bertics & Gill, 1985; Gill *et al.*, 1987). Subsequent studies using site-directed mutagenesis and C-terminal deletions have supported a model in which the C-terminus acts as an autoinhibitory domain whose effects can be relieved via phosphorylation (Bertics *et al.*, 1988; Honegger *et al.*, 1988; Walton *et al.*, 1990).

Previous investigations have shown that polyamines, basic poly(amino acids) and proteins containing basic amino acid domains can modulate the insulin receptor tyrosine kinase (Rosen & Lebowitz, 1988; Sacks & McDonald, 1988; Fujita-Yamaguchi *et al.*, 1989a; Kohanski, 1989; Morrison *et al.*, 1989) and several other serine/threonine and tyrosine kinases (Walton & Gill, 1981; Tabor & Tabor, 1984; Ahmed *et al.*, 1986; Palczewski *et al.*, 1989). Similarly, poly(L-lysine) was found to enable the EGF receptor to readily phosphorylate acetyl-lipocortin 1, which is only poorly phosphorylated in the absence of poly(L-lysine) *in vitro* (Abdel-Ghany *et al.*, 1989). In addition, protamine has

been reported to increase EGF binding (Huang *et al.*, 1984; Lokeshwar *et al.*, 1989) and EGF-mediated growth (Neufeld & Gospodarowicz, 1987; Lokeshwar *et al.*, 1989). However, the mechanisms by which poly(L-lysine) and protamine produce these effects is not fully understood.

The general stimulatory effect of basic proteins on tyrosine and serine/threonine kinases suggests a fundamental role for proteins containing basic domains in kinase substrate recognition and/or regulation. These domains could alter EGF receptor tyrosine kinase activity through a variety of mechanisms. For example, they may modify EGF binding or EGF-dependent receptor activation, or they may directly increase the catalytic capacity (V_{max}) of the enzyme, similar to the mechanism of action of EGF itself (Pike *et al.*, 1982; Erneux *et al.*, 1983). Alternatively, they may modify substrate binding, thereby leading to changes in both the V_{max} and the Michaelis constants (K_m values) of the receptor. Therefore we have further explored the influence of basic poly(amino acids) and protamine on EGF receptor kinase activity and investigated the mechanism by which these effectors modulate receptor action. Poly(L-lysine), poly(L-arginine) and protamine were found to stimulate EGF receptor kinase activity 3–5-fold. EGF dose–response studies indicated that the stimulation was not due to an altered responsiveness of the receptor to EGF. Instead, kinetic analysis revealed that the basic proteins produced an increase in the V_{max} and a decrease in the apparent K_m for the peptide substrate, angiotensin II, leading to an overall increase in the V_{max}/K_m ratio. Further kinetic studies with the EGF receptor demonstrated that protamine and poly(L-lysine) changed the pattern of product inhibition by ADP from competitive to non-competitive with angiotensin II. These observations strongly suggest that the basic effectors stimulate EGF receptor kinase activity by altering the binding of reactants. In addition, the C-terminal region of the EGF receptor, which appears to be involved in substrate recognition (Margolis *et al.*, 1990), may also be involved

Abbreviations used: EGF, epidermal growth factor.

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in the effects of basic proteins, since a C-terminally truncated receptor, lacking the four major self-phosphorylation sites, exhibits a decreased response to these compounds when compared with that measured for the wild-type receptor. These results illustrate the complex nature of EGF receptor/substrate interactions and suggest that intracellular factors, such as basic proteins or proteins with basic domains, can have an important effect on EGF receptor kinase activity.

EXPERIMENTAL

Materials

Angiotensin II (DRVYIHPF), poly(L-glutamic acid) (sodium salt; molecular mass 2–15 kDa; average 10 kDa), poly(L-lysine) (hydrochloride; molecular mass 15–30 kDa; average 20 kDa), poly(L-arginine) (hydrochloride; molecular mass 15–70; average 40 kDa), and protamine chloride (from salmon sperm) were purchased from Sigma. *src*-peptide (RRLIEDAEYARG) was obtained from Peninsula Laboratories (Belmont, CA, U.S.A.); EGF was from Upstate Biotechnology, Lake Placid, NY, U.S.A.; and [γ - 32 P]ATP (3000 Ci/mmol) was from Amersham.

Preparation of cell membranes and purified EGF receptor

Cell membranes were prepared as described previously (Bertics *et al.*, 1988) from human A431 epidermoid carcinoma cells. Cells were grown to near confluence on 15 cm plates, washed with ice-cold Ca^{2+} -free phosphate-buffered saline (145 mM-NaCl/10 mM-sodium phosphate, pH 7.4) containing 2.5 mM-EDTA and -EGTA, scraped from plates with a rubber policeman and pelleted by centrifugation (1500 *g*, 10 min). The cells were lysed in a 20 mM-Hepes buffer, pH 7.4, containing 1.5 mM-MgCl₂, 2.5 mM-EDTA and -EGTA, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-dithiothreitol, 4 mM-benzamidine, 0.1 trypsin-inhibitory unit of aprotinin/ml and 20 μ g of leupeptin/ml. The cells were homogenized and sonicated, and membranes were prepared by differential centrifugation as described previously (Bertics *et al.*, 1988). The final membrane pellet was resuspended in a 20 mM-Hepes buffer, pH 7.4, containing 1 mM-dithiothreitol, 10 μ g of leupeptin/ml and 1 mM-EGTA.

Purified EGF receptor was isolated from human A431 or mouse B82L cells, which contain the mutant human EGF receptor truncated at amino acid 1023 (Chen *et al.*, 1989; Walton *et al.*, 1990), by immunoaffinity chromatography as detailed earlier (Weber *et al.*, 1984; Gill & Weber, 1987). In brief, cells were isolated as described above and suspended in a 20 mM-Hepes buffer, pH 7.4, containing 5 mM-EGTA, 1% Triton X-100, 10% glycerol, 0.1 mM-Na₃VO₄, 5 mM-NaF, 4 mM-PP_i, 4 mM-benzamidine hydrochloride, 0.08 mg of aprotinin/ml, 1 mM-dithiothreitol, 0.04 mg of leupeptin/ml and 1 mM-phenylmethanesulphonyl fluoride. The cells were sonicated and then centrifuged for 10 min at 25000 *g*. The supernatant, containing the Triton X-100-solubilized EGF receptor, was incubated with the anti-(EGF receptor) 528 monoclonal antibody coupled to CNBr-activated Sepharose 4B beads. This antibody recognizes the EGF-binding site (Gill *et al.*, 1984). The EGF receptor was eluted from the antibody beads as an active tyrosine kinase using 5 μ M-EGF. The final receptor solution contained 20 mM-Hepes, pH 7.4, 1 mM-EDTA, 130 mM-NaCl, 0.05% Triton X-100, 10% glycerol, 1 mM-dithiothreitol and 5 μ M-EGF. All of the above procedures were done at 0–4 °C. The receptor was highly purified, as assessed by SDS/PAGE and N-terminal sequence analysis (Weber *et al.*, 1984).

Assay of EGF receptor tyrosine kinase activity

In general, the protein-tyrosine kinase activity of the purified EGF receptor was measured in reaction mixtures containing

20 mM-Hepes, pH 7.4, 2 mM-MnCl₂, 50 μ M-Na₃VO₄, 0.5 mg of BSA/ml, 0.5% aprotinin, 10–200 nM-EGF receptor (> 200 nM-EGF) and the indicated concentrations of [γ - 32 P]ATP [(1–3) $\times 10^4$ c.p.m./pmol], exogenous substrates and/or basic effectors. Unless otherwise indicated, the reaction was carried out in a total volume of 60 μ l for 10 min at 4 °C or for 5 min at 30 °C. Phosphorylation of angiotensin II was terminated by the addition of 20 μ l of 25% trichloroacetic acid. Carrier protein (80 μ g of BSA) was added and peptide phosphorylation was measured using the phosphocellulose binding assay (Bertics & Gill, 1985). Self-phosphorylation was analysed by the filter binding assay (Weber *et al.*, 1984; Bertics & Gill, 1985). When cell membranes were used as the source of protein-tyrosine kinase activity, the endogenous EGF receptor was activated by incubation with 300 nM-EGF for 45 min at 4 °C, followed by 15 min at room temperature, before initiating the reaction. Control incubations contained all assay components except the EGF receptor.

RESULTS

Stimulation of EGF receptor kinase activity by basic proteins

Previous studies have shown that basic proteins and polyamines can influence the activity of certain protein-serine/threonine and -tyrosine kinases (Walton & Gill, 1981; Fujita-Yamaguchi *et al.*, 1989a; Palczewski *et al.*, 1989), but the mechanism of this effect is not fully known. To assess how the tyrosine kinase activity of the EGF receptor is modulated by these agents, we examined the dose-dependent ability of poly(L-lysine), protamine, poly(L-arginine) and poly(L-glutamate) to affect angiotensin II phosphorylation. Fig. 1 shows that maximum stimulation of the EGF receptor kinase was observed at approx. 5 μ M-poly(L-lysine) (100 μ g/ml), 10 μ M-protamine (200 μ g/ml) and 0.3 μ M-poly(L-arginine) (10 μ g/ml), whereas poly(L-glutamate), at concentrations up to 1 mM, had no effect.

Fig. 1. Modulation of purified EGF receptor protein-tyrosine kinase activity by basic poly(amino acids) and protamine

Immunoaffinity-purified EGF receptor (20–40 nM; 800 nM-EGF) was incubated with 1 mM-angiotensin II and the indicated final concentrations of poly(L-lysine) (●), protamine (□), poly(L-arginine) (■) or poly(L-glutamate) (○) prior to initiating the reaction with 10 μ M-[γ - 32 P]ATP [(1–3) $\times 10^4$ c.p.m./pmol] as described in the Experimental section. The assays were performed at 4 °C for 10 min, and angiotensin II phosphorylation was measured using the phosphocellulose binding assay. The fold stimulation represents the reaction velocity (pmol of phosphate incorporated/min) in the presence of effector divided by the velocity in the absence of effector. Each point is the mean $\pm$ S.E.M. ($n = 4$ –16).

Fig. 2. Effect of poly(L-lysine) on the EGF dose-response curve for the activation of receptor protein-tyrosine kinase activity

Cell membranes containing 70 μ g of protein were preincubated with the indicated concentrations of EGF as described in the Experimental section. (a) The angiotensin II (1 mM) phosphorylation assay included 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ [$(1\text{--}3) \times 10^4$ c.p.m./pmol], either with (●) or without (○) 5 μ M-poly(L-lysine). (b) The effect of EGF on kinase activity in the absence of poly(L-lysine), as shown in (a), is replotted on a different scale to demonstrate the similarity of EGF activation in the presence and absence of basic effector. Each point is the mean $\pm$ range. Similar results were obtained in two different experiments.

The EGF receptor kinase activation is biphasic, with increasing concentrations of poly(L-lysine) and poly(L-arginine) eventually leading to a loss of stimulation. High levels of poly(L-arginine) actually inhibited angiotensin II phosphorylation. Conversely, protamine did not lead to a loss of angiotensin II phosphorylation

at the concentrations tested. Control experiments demonstrated that the effectors do not increase binding of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to the phosphocellulose papers used to assess EGF receptor activity, nor do the effectors appear to contain any contaminating substrates for the EGF receptor (results not shown). Also, since the basic polypeptides and protamine contain Cl^- as the counter-ion, we determined whether the counter-ion was responsible for the observed stimulation of EGF receptor kinase activity. At the levels of the effectors giving maximum stimulation of kinase activity, the concentration of Cl^- present was 0.05–2 mM. However, Cl^- from three different sources (NaCl, KCl and NH_4Cl) lacked any stimulatory effect when tested at concentrations ranging from 0.05 to 5 mM, suggesting that the effects of the basic polyamino acids and protamine are not due to the Cl^- counter-ion (results not shown). Therefore the basic nature of the effectors appears to be important for the increase in EGF receptor kinase activity.

Effect of Poly(L-lysine) on EGF receptor activation by EGF

The basic proteins may modulate EGF receptor kinase activity by altering EGF binding or EGF-dependent activation of the receptor. Therefore the dose-dependent activation of the membrane-associated receptor by EGF was analysed in the presence of poly(L-lysine) (Fig. 2). The basal and EGF-stimulated levels of kinase activity increased by an equivalent degree (4-fold). In addition, the ED_{50} (concn. causing half-maximal stimulation) for EGF, determined using non-linear regression analysis, was similar in the absence and in the presence of poly(L-lysine) (150 and 140 nM respectively). Therefore poly(L-lysine) did not substantially alter the receptor's responsiveness to EGF, suggesting an alternative mechanism for its stimulation of kinase activity.

Basic effector stimulation of *src*-peptide and EGF receptor self-phosphorylation

To explore whether the basic effectors modify kinase activity specifically by interacting with the peptide substrate, angiotensin II, or directly with the EGF receptor itself, we investigated the effectors' ability to alter both receptor self-phosphorylation and the phosphorylation of a different peptide substrate, i.e. *src*-peptide (a synthetic peptide corresponding to a self-phosphorylation site in p60^{src}). Table 1 illustrates that under initial velocity conditions (30 s, 4 $^\circ\text{C}$, 0.2 μ M-ATP), poly(L-lysine) and protamine stimulated self-phosphorylation by approx. 2.6- and 1.7-fold respectively in the absence of exogenous substrate.

Table 1. Substrate- and temperature-dependence of stimulation of the EGF receptor kinase by basic effectors

Purified EGF receptor (10–200 nM; ≥ 800 nM-EGF) was mixed with the indicated final concentrations of basic poly(amino acids) or protamine prior to initiating the reaction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The self-phosphorylation assays were carried out at 4 $^\circ\text{C}$ for 0.5 min using 0.2 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^4 c.p.m./pmol) or for 10 min using 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^4 c.p.m./pmol). The phosphorylation of 0.2 mM-*src*-peptide was measured with 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^4 c.p.m./pmol) for 10 min at 4 $^\circ\text{C}$. The phosphorylation of 1 mM-angiotensin II was performed with 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1×10^4 c.p.m./pmol) for 10 min at 4 $^\circ\text{C}$ or for 2 min at 30 $^\circ\text{C}$ as indicated. The fold activation is the reaction velocity in the presence of effector divided by the velocity in the absence of effector, both determined under the reaction conditions indicated. Each value is the mean $\pm$ S.E.M. ($n = 4$).

Substrate	Assay conditions	Activation of EGF receptor kinase activity (fold)		
		5 μ M-Poly(L-Lys)	10 μ M-Protamine	0.3 μ M-Poly(L-Arg)
EGF receptor	0.5 min	2.6 $\pm$ 0.2	1.7 $\pm$ 0.0	1.1 $\pm$ 0.1
	10 min	3.1 $\pm$ 0.3	2.8 $\pm$ 0.2	1.3 $\pm$ 0.0
<i>src</i> -peptide	—	3.0 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1
Angiotensin II	4 $^\circ\text{C}$	4.0 $\pm$ 0.1	2.0 $\pm$ 0.2	1.8 $\pm$ 0.2
	30 $^\circ\text{C}$	2.3 $\pm$ 0.1	1.8 $\pm$ 0.1	2.0 $\pm$ 0.1

Fig. 3. Determination of kinetic constants for EGF receptor phosphorylation of angiotensin II in the presence of basic effectors

The assays were carried out for 5 min at 30 °C using 10–20 nM-EGF receptor (300–500 nM-EGF) as described in the Experimental section. The reaction mixtures contained no addition (○), 5 μM-poly(L-lysine) (▲), 10 μM-protamine (△) or 0.3 μM-poly(L-arginine) (●) as indicated. (a) The reactions contained 0.05–2 mM-angiotensin II and 10 μM-ATP (similar results were obtained with 20 μM-ATP). (b) Double-reciprocal plot of $1/v$ versus $1/[angiotensin\ II]$ data from (a). (c) The assays were performed with 3 mM-angiotensin II and 2–20 μM-ATP. (d) Double-reciprocal plot of $1/v$ versus $1/[ATP]$ data from (c). Insets in (b) and (d) are expanded views of the regions where the lines cross the x-axis. Similar results were obtained in 2–6 separate experiments.

Poly(L-arginine) had little detectable effect under these conditions. Under otherwise maximal phosphorylation conditions (10 min, 4 °C, 10 μM-ATP), poly(L-lysine) had a similar effect to that seen at 30 s, while protamine was more stimulatory and poly(L-arginine) slightly increased self-phosphorylation. Poly(L-glutamate) had no measurable effect under either initial velocity or maximal phosphorylation conditions. The basic effectors were also capable of stimulating the phosphorylation of *src*-peptide by 1.4–3.0-fold (Table 1). Thus the increase in EGF receptor self-phosphorylation and *src*-peptide phosphorylation by the basic effectors suggests that the effectors can affect the receptor itself and are not specific for angiotensin II.

Influence of temperature on kinase stimulation by basic effectors

Because the preceding experiments were performed at 4 °C, it was of interest to determine the temperature-dependence of the stimulatory effect. Angiotensin II phosphorylation was measured at 4 and 30 °C in the presence or the absence of an optimal concentration of poly(L-lysine), poly(L-arginine) or protamine. As illustrated in Table 1, poly(L-lysine) was less effective at stimulating kinase activity at 30 °C, but still yielded an increase comparable with that observed with poly(L-arginine) and protamine. Furthermore there appeared to be no change in the concentration-dependence of poly(L-lysine) stimulation at 30 °C (results not shown). Protamine and poly(L-arginine) had similar effects at both temperatures. Thus the stimulation seen with these agents was relatively temperature-independent, and may reflect

the ability of basic regions of proteins to similarly affect EGF receptor kinase activity *in vivo*.

Determination of the kinetic constants for EGF receptor phosphorylation of angiotensin II

To investigate how these basic effectors alter EGF receptor catalytic activity, we first studied their influence on the kinetic parameters of angiotensin II phosphorylation (V_{max} , K_m and V_{max}/K_m). Fig. 3 and Table 2 show that poly(L-lysine) increased the V_{max} at least 2-fold when the concentration of angiotensin or ATP was varied. Protamine and poly(L-arginine) had less of an effect: the V_{max} increased approx. 20–40% when angiotensin II and ATP were varied. All the effectors lowered the apparent K_m for angiotensin II by 40–50% and had variable but smaller effects on the K_m of ATP. Poly(L-lysine) slightly increased, protamine had no effect on, and poly(L-arginine) slightly decreased the K_m of ATP. In general, all of the effectors increased V_{max} and V_{max}/K_m , suggesting that these effectors are modifying the binding or orientation of the reactants in the enzyme active site, thereby leading to an increase in the catalytic efficiency of the EGF receptor.

Alteration of the EGF receptor kinetic mechanism by protamine

Since the basic effectors appeared to alter the kinetic parameters of angiotensin II phosphorylation, product inhibition studies were performed to investigate whether these agents could be inducing a shift in the order or preference of reactant addition

Table 2. Kinetic analysis of the influence of basic effectors on EGF receptor protein-tyrosine kinase activity

The kinetic constants were determined from the data in Fig. 3 by non-linear regression analysis (Greco *et al.*, 1982) and are reported as means $\pm$ S.E.M. ($n = 4-5$). The units of $V_{\max}$ are pmol of phosphate incorporated into angiotensin II/min. The units of K_m are mM for angiotensin II and μ M for ATP. The ' $V_{\max}/K_m$ effect' is the $V_{\max}/K_m$ determined in the presence of the basic effector divided by the $V_{\max}/K_m$ value found in the absence of the effector, expressed as a percentage.

Substrate varied	Basic effector	$V_{\max}$ (pmol/min)	K_m (mM or μ M)	$V_{\max}/K_m$	$V_{\max}/K_m$ effect (%)
Angiotensin II	—	5.04 ± 0.44	1.15 ± 0.13	4.38 ± 0.63	100
	Poly(L-Lys)	10.9 ± 0.95	0.67 ± 0.08	16.3 ± 2.4	372
	Protamine	6.36 ± 0.67	0.57 ± 0.09	11.2 ± 2.1	256
	Poly(L-Arg)	6.10 ± 0.44	0.64 ± 0.07	9.53 ± 1.2	218
ATP	—	4.62 ± 0.95	4.68 ± 1.04	0.99 ± 0.02	100
	Poly(L-Lys)	11.3 ± 0.5	5.85 ± 0.37	1.94 ± 0.04	196
	Protamine	6.28 ± 1.22	4.68 ± 1.40	1.39 ± 0.15	140
	Poly(L-Arg)	6.38 ± 1.23	3.18 ± 0.83	2.05 ± 0.15	207

Table 3. Kinetic constants for inhibition by ADP of EGF receptor phosphorylation of angiotensin II

The assays were carried out for 5 min at 30 °C using 6–12 nM-EGF receptor (≥ 600 nM-EGF) as described in the Experimental section. The reaction mixtures contained 0–90 μ M-ADP and 10 μ M-protamine as indicated. The kinetic constants were determined by non-linear regression analysis (Greco *et al.*, 1982) and are reported as the means of multiple determinations $\pm$ S.E.M. ($n = 3-6$), each computed as a value $\pm$ S.E.M. ($n = 12-16$).

Protamine	Substrate varied	Inhibition pattern	$K_{i(\text{slope})}$ (μ M)	$K_{i(\text{intercept})}$ (μ M)
—	AII*	Competitive	14.3 ± 1.3	—
—	ATP†	Competitive	9.35 ± 1.96	—
+	AII*	Non-competitive	44.5 ± 9.9	67.1 ± 21.7
+	ATP†	Competitive	7.83 ± 2.88	—

* The reactions contained 0.4–2 mM-angiotensin II and 10 μ M-ATP.

† The assays were performed with 1 mM-angiotensin II and 2–10 μ M-ATP.

to the EGF receptor, i.e. the kinetic mechanism. Table 3 shows that ADP acts as a competitive inhibitor with both angiotensin II and ATP in the absence of protamine, suggesting two possible reaction mechanism, Random Bi Bi or equilibrium ordered, where the steps between substrate and ADP binding are at thermodynamic equilibrium (Cleland, 1977). Initial velocity patterns were used to determine the actual mechanism, since the models predict that the lines on a double-reciprocal plot would intersect on the vertical axis for an equilibrium ordered mechanism ($K_a \gg K_m$), but not for a Random Bi Bi mechanism. The initial velocity data fit best to the Random Bi Bi model (Fig. 4). Therefore, in the absence of protamine, the order of addition of ATP and angiotensin II and the release of ADP and phosphorylated angiotensin II do not appear obligatory. Also, since ADP is a competitive inhibitor with angiotensin II, their binding must be mutually exclusive, i.e. the enzyme–angiotensin II–ADP ternary complex cannot form. It should be noted that a previous kinetic study of the EGF receptor kinase found the reaction mechanism to be preferentially Ordered Bi Bi, with the binding of a different exogenous peptide substrate preceding that of ATP, followed by catalysis and the release of phosphorylated peptide substrate and then ADP (Erneux *et al.*, 1983). Although we observed a random mechanism, there may be a preferred order of substrate addition such that angiotensin II binding

tends to precede that of ATP. The difference between the two mechanisms may be due to the use of different exogenous substrates to determine the kinetic mechanism. For example, some peptide substrates may bind to the enzyme much more readily than ATP, indicating an apparent Ordered Bi Bi reaction mechanism. Other substrates may not bind to the enzyme as efficiently, or their binding may be facilitated by ATP addition, thereby resulting in kinetic patterns indicating a Random Bi Bi mechanism.

ADP is competitive with angiotensin II in the absence of protamine, but non-competitive in its presence (Table 3). This inhibition pattern fits a Random Bi Bi mechanism where the enzyme–angiotensin II–ADP complex can form, or an ordered reaction in which ATP binding precedes that of angiotensin II, followed by catalysis and the ordered release of products. We have yet to find a competitive inhibitor of angiotensin II binding that could be used to determine which of the above mechanisms is correct, i.e. a competitive inhibitor of angiotensin II binding would be either competitive or non-competitive with ATP in a random mechanism, but would be uncompetitive with ATP in the ordered reaction. We have tried sarmesin (a non-phosphorylatable angiotensin II analogue; Goghari *et al.*, 1986) and tyrphostin (a synthetic compound reported to be a competitive inhibitor of protein substrate binding to the EGF receptor; Gazit *et al.*, 1989), but both were found to be non-competitive with angiotensin II in the presence of protamine (results not shown). Regardless of the precise reaction mechanism occurring in the presence of protamine, the central issue is the ability of the receptor to form the enzyme–angiotensin II–ADP ternary complex in the presence of protamine but not in its absence. A similar change in the capacity to form the ternary complex was also seen with poly(L-lysine) (results not shown). These results suggest that the basic effectors can alter reactant binding to the EGF receptor.

Kinase activity of purified intact EGF receptor and a C-terminally truncated receptor in the presence of basic proteins

The C-terminus of the EGF receptor, which acts as a competitive inhibitor of exogenous substrate phosphorylation (Bertics & Gill, 1985; Gill *et al.*, 1987; Bertics *et al.*, 1988), is a region that may interact with the basic effectors. This region contains the four primary sites of self-phosphorylation, Tyr residues 1173, 1148, 1086 and 1068 (Downward *et al.*, 1984; Margolis *et al.*, 1989), and appears to interact with the tyrosine-phosphate binding, *src*-like (e.g. SH2) domains of EGF receptor substrates such as the GTPase-activating protein and phospholipase C- γ (Margolis *et al.*, 1990). Truncations in this region of the EGF receptor (Khazaie *et al.*, 1988; Haley *et al.*, 1989) and

Fig. 4. Initial velocity patterns of EGF receptor tyrosine kinase activity as a function of angiotensin II and ATP concentrations

The reactions were carried out as described in the Experimental section for 5 min at 30 °C in the presence of 2 nM purified EGF receptor (1.2 μM-EGF). (a) Double-reciprocal plot of $1/v$ versus $1/[\text{angiotensin II}]$ with 2 (○), 3 (●), 5 (□) and 10 (■) μM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^4 c.p.m./pmol). (b) Double-reciprocal plot of $1/v$ versus $1/[\text{ATP}]$ with 0.4 (○), 0.6 (●), 1 (□) and 2 (■) mM-angiotensin II. Non-linear regression analysis (Greco *et al.*, 1982) indicated that the data fit best to the Random Bi Bi reaction mechanism. Therefore this model was used to obtain the lines plotted in each panel. Similar results were found in two different experiments.

v-erbB (Beug *et al.*, 1986; Gamett *et al.*, 1986; Raines *et al.*, 1988), whose gene product has sequence similarity to the EGF receptor kinase domain (Maihle & Kung, 1988), strongly correlate with their capacity to transform fibroblasts rather than erythroblasts, suggesting that this region is important in determining specific cellular effects. Our findings suggest that the strong negative charges of the self-phosphorylated tyrosines in this region may contribute to the specific stimulation of EGF receptor kinase activity by the cationic groups of the basic proteins. Therefore we assessed the role of the EGF receptor C-terminus in basic protein modulation of kinase activity by comparing angiotensin II phosphorylation using the intact receptor with that observed with a purified mutant EGF receptor that is C-terminally truncated at amino acid 1023, and thus lacks the four primary self-phosphorylation sites (Chen *et al.*, 1989; Walton *et al.*, 1990). Table 4 shows that stimulation of this mutant kinase by poly(L-lysine) was substantially decreased, while protamine and poly(L-arginine) appear to be ineffective at influencing angiotensin II phosphorylation by the immunoaffinity-purified truncated receptor. These results suggest

Table 4. Effect of C-terminal truncation of the EGF receptor on modulation of its kinase activity by the basic effectors

Purified wild-type EGF receptor or C-terminally truncated EGF receptor (20–40 nM; 800 nM-EGF) was mixed with the indicated basic effector before initiating the reaction with 10 μM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1×10^4 c.p.m./pmol). The phosphorylation assay was carried out as described in the Experimental section using 1 mM-angiotensin II as the exogenous substrate. The fold activation is the reaction velocity in the presence of effector divided by the velocity in the absence of effector using either the wild-type or C-terminally truncated EGF receptor as indicated for both measurements. The data are shown as the means $\pm$ S.E.M. ($n = 6\text{--}10$).

Effector	Activation of EGF receptor kinase activity (fold)	
	Wild-type receptor	C-Terminally truncated receptor
5 μM-Poly(L-Lys)	4.5 ± 0.7	2.4 ± 0.2
10 μM-Protamine	3.0 ± 0.1	0.95 ± 0.25
0.3 μM-Poly(L-Arg)	2.6 ± 0.1	0.88 ± 0.16
50 μM-Poly(L-Glu)	0.97 ± 0.05	1.1 ± 0.3

that the C-terminus of the receptor may be involved, at least in part, in the modulation of EGF receptor kinase activity by basic proteins.

DISCUSSION

In the present study, the protein-tyrosine kinase activity of immunoaffinity-purified EGF receptor was found to be stimulated by poly(L-lysine), poly(L-arginine) and protamine. The cationic nature of the poly(amino acid)/protein activators appears to be involved in their mechanism of action, since kinase activity was not affected by either the acidic poly(amino acid) poly(L-glutamate) or the Cl^- counter-ion. The effects of the basic poly(amino acids) and protamine appear to be specific, since low concentrations of poly(L-lysine), poly(L-arginine) and protamine (5, 0.3 and 10 μM respectively) maximally stimulated angiotensin II phosphorylation. There are several possible modes of tyrosine kinase stimulation by these effectors, including effects on the EGF dose-dependent activation of the receptor, the V_{max} of the reaction and the process of substrate binding/catalysis. While there was little effect on the EGF dose-dependent activation of the receptor, the addition of basic effectors resulted in a decrease in the K_m for angiotensin II and an increase in the V_{max}/K_m ratio, suggesting that these agents can influence the binding of the peptide substrate to the receptor. The stimulation of phosphorylation by the basic effectors, however, is not specific to angiotensin II, since they also increased *src*-peptide phosphorylation and receptor self-phosphorylation. Thus the basic effectors appear to be able to interact directly with the EGF receptor to bring about changes in reactant binding and catalytic efficiency.

The ADP inhibition studies revealed that protamine and poly(L-lysine) shift the competitive inhibition of angiotensin II phosphorylation by ADP to a non-competitive pattern. This inhibition pattern is consistent with a mechanism where the enzyme-angiotensin II-ADP ternary complex can form, such as Random Bi Bi or Ordered Bi Bi where ATP binds first and ADP is released last. Therefore protamine and poly(L-lysine) alter angiotensin II and ADP binding such that they can both be present on the enzyme at the same time. This result, together with the observed decrease in the K_m for angiotensin II and the increase in the V_{max}/K_m ratio, demonstrates that the altered

reactant binding and catalytic efficiency of the receptor kinase is probably due to a change in the orientation of the reactants or the conformation of the EGF receptor active site.

Because EGF receptor substrates are typically large proteins, enzyme-substrate interaction probably involves multiple points of contact which help to establish a favourable substrate orientation or conformation for phosphorylation. It is conceivable that the effects on peptide phosphorylation found in the present study are a result of mimicking this type of interaction. For example, small substrates such as angiotensin II and *src*-peptide lack the basic amino acids often found near tyrosine phosphorylation sites and the basic domains located distal to the phosphorylation sites of many larger substrates. This lack of basic domains could contribute to the comparatively high K_m values found for peptide substrates. Basic domains could affect substrate recognition and phosphorylation by interacting with specific regions of the EGF receptor, e.g. the negatively charged sequence involved in EGF receptor internalization and ligand-stimulation of cytosolic Ca^{2+} (CAIN domain; Chen *et al.*, 1989) and/or the negatively charged self-phosphorylation domain. This type of interaction has been found to occur between the self-phosphorylated sites and the *src*-like regions (e.g. SH2) of various proteins, including several EGF receptor substrates (Margolis *et al.*, 1990, Koch *et al.*, 1991). EGF receptor substrates containing basic domains that may modulate their phosphorylation include the *ras* GTPase activator protein (Vogel *et al.*, 1988; Ellis *et al.*, 1990), various cytoskeletal proteins such as ezrin (Gould *et al.*, 1989) and protein 4.1 (Subrahmanyam *et al.*, 1991), and the c-Ki-*ras* 2 gene product. Substrates lacking these basic regions could have other modulatory proteins fulfilling this role. Thus the basic regions of EGF receptor substrates or other modulatory proteins may act as part of a system of multiple points of contact on the receptor which enhance the substrate's ability to interact with the EGF receptor and thereby modulate substrate recognition.

We have found that the stimulation of angiotensin II phosphorylation by the basic effectors is decreased or lost when using a C-terminally truncated EGF receptor missing the primary sites of self-phosphorylation. Our earlier work has shown that these self-phosphorylation sites act as competitive/alternate substrates for the kinase (Bertics & Gill, 1985; Gill *et al.*, 1987; Bertics *et al.*, 1988). Further studies have found that self-phosphorylation removes this region of the receptor from the kinase domain and allows better access for exogenous substrates (Bertics *et al.*, 1988; Honegger *et al.*, 1988; Walton *et al.*, 1990). However, the reversibility of the self-phosphorylation reaction (Hubler *et al.*, 1989) indicates that the phosphorylated tyrosine residues of the receptor can still be recognized as substrates. Therefore the basic amino acids or domains of cellular substrates may affect substrate recognition by interacting with the highly negatively charged C-terminal self-phosphorylated sites. This interaction not only may increase substrate-binding affinity, but it may also block the re-association of the self-phosphorylated sites with the kinase domain. Hindering this re-association would result in increased accessibility of the kinase to exogenous substrates/products, such as we observed in the present study.

The influence of basic effectors is not limited to the EGF receptor. In particular, insulin receptor phosphorylation of calmodulin illustrates the ability of basic domains to modulate tyrosine kinase activity. The insulin receptor phosphorylates calmodulin *in vivo*, but *in vitro* calmodulin phosphorylation requires basic cofactors such as poly(L-lysine) and protamine (Sacks & McDonald, 1988). Insulin receptor kinase phosphorylation of the human c-Ki-*ras* 2 gene product, however, does not require these basic proteins. Instead, this substrate contains a basic domain that may serve a function similar to that of the calmodulin cofactors. In fact, a synthetic peptide cor-

responding to this basic domain was found to induce insulin receptor phosphorylation of calmodulin *in vitro* in the absence of poly(L-lysine) or protamine (Fujita-Yamaguchi *et al.*, 1989b). Thus the effects of basic proteins may reflect the general importance of basic domains of proteins in determining substrate specificity. The changes in the kinetic properties of the EGF receptor that we have found in this study and the importance of the C-terminus in mediating the effects of basic proteins should prove useful for understanding the means by which basic domains or proteins modulate substrate phosphorylation.

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