The EMBO Journal vol.7 no.3 pp.707 – 710, 1988

A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction

W.H.Moolenaar[4], A.J.Bierman, B.C.Tilly, I.Verlaan, L.H.K.Defize, A.M.Honegger[2], A.Ullrich[1] and J.Schlessinger[2,3]

The Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, [1]Genentech, Inc., South San Francisco, CA 94080 and [2]Rorer Biotechnology, Inc., 4 Research Court, Rockville, MD 20850, USA

[3]On leave from the Weizmann Institute of Science, Rehovot, Israel

[4]Present address: Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands

Communicated by J.Schlessinger

The EGF-receptor (EGF-R) is a transmembrane glycoprotein with intrinsic protein tyrosine kinase (TK) activity. To explore the importance of the receptor TK in the action of EGF, we have used transfected NIH-3T3 cells expressing either the normal human EGF-R or a receptor mutated at Lys721, a key residue in the presumed ATP-binding region. The wild-type receptor responds to EGF by causing inositol phosphate formation, Ca^{2+} influx, activation of Na^+/H^+ exchange and DNA synthesis. In contrast, the TK-deficient mutant receptor fails to evoke any of these responses. It is concluded that activation of the receptor TK is a crucial signal that initiates the multiple post-receptor effects of EGF leading to DNA synthesis. Furthermore, the results suggest that tyrosine phosphorylation plays a role in the activation of the phosphoinositide signalling system.

Key words: EGF-receptor/ATP-binding/signal transduction/point mutation/tyrosine kinase

Introduction

The binding of epidermal growth factor (EGF) to its cell surface receptor initiates a variety of biochemical and physiological changes in the target cell, leading to enhanced DNA synthesis and cell division (reviewed in Carpenter and Cohen, 1979; Schlessinger, 1986). The EGF-receptor (EGF-R) is a 170-kd transmembrane glycoprotein with an intrinsic protein tyrosine kinase (TK) domain. EGF binding activates the TK leading to receptor autophosphorylation and to the phosphorylation of specific substrates (reviewed in Carpenter and Cohen, 1979; Hunter and Cooper, 1985; Schlessinger, 1986). While recent data suggest that this TK activity regulates receptor degradation after endocytosis (Honegger *et al.*, 1987a,b), uncertainty still exists about its role in mediating the various cellular responses to EGF. Among the immediate consequences of receptor activation are: (i) a rise in cytoplasmic free Ca^{2+} (Moolenaar *et al.*, 1986), (ii) activation of Na^+/H^+ exchange resulting in intracellular alkalinization (Rothenberg *et al.*, 1983; Moolenaar, 1986) and, at least in some cell types, (iii) activation of the phosphoinositide-second messenger system (Sawyer and Cohen, 1981; Pike and Eakes, 1987; Tilly *et al.*, 1988). To explore the importance of the receptor TK activity in EGF action, we have used transfected NIH-3T3 cells expressing either wild-type human EGF-R or a mutant receptor containing an alanine substituted for Lys721, a key residue in the presumed ATP-binding domain. We have previously shown that this receptor mutant is devoid of protein TK activity both *in vitro* and *in vivo* (Honegger *et al.*, 1987a,b). Unlike wild-type EGF-R, which undergoes typical internalization and degradation, the TK-defective mutant recycles back to the cell surface after internalization while bound EGF molecules undergo degradation (Honegger *et al.*, 1987a). Moreover, the mutant EGF-R is unable to stimulate proto-oncogenes c-*myc* and c-*fos* expression and DNA synthesis (Honegger *et al.*, 1987b). Here we show that this TK-defective EGF-R mutant is unable to stimulate inositol phosphate formation, Ca^{2+} influx and Na^+/H^+ exchange, while wild-type EGF-R is able to stimulate these responses in the transfected cells. On the basis of these results, we propose that the protein TK activity of EGF-R is essential for signal transduction mediated by EGF leading to DNA synthesis.

Results and discussion

In order to establish the role for the protein TK function of the EGF-R as a mediator of the pleiotropic response of EGF, we have prepared a TK-defective EGF-R mutant by *in vitro* site-directed mutagenesis in which Lys721 was replaced by an alanine residue (Honegger *et al.*, 1987a,b). Several cell lines expressing either wild-type EGF-R or the mutant receptor K721A were generated. The NIH-3T3 clone used for transfections has previously been shown to lack detectable amounts of endogenous murine EGF-R (Honegger *et al.*, 1987a,b). Full characterization of the transfected NIH-3T3 cells expressing either wild-type or mutant EGF-R was described previously (Honegger *et al.*, 1987a,b). Briefly, both wild-type and mutant receptors are properly processed and they are displayed on the cell surface with typically high and low affinity binding sites. Similar to the wild-type receptor, phorbol ester (TPA) abolishes the high affinity binding sites of mutant receptors. However, unlike the wild-type receptor, the mutant receptor does not exhibit EGF-sensitive protein TK activity and is unable to undergo autophosphorylation and to phosphorylate endogenous substrates *in vitro* (Honegger *et al.*, 1987a,b), in living cells (Honegger *et al.*, 1987a) and in membrane preparations (Figure 1). For the present study, we have chosen transfected cell lines, which express similar amounts of either wild-type EGF-R (HER14 cells) or mutant EGF-R (K721A cells). Both cell lines display on their cell surface ~ 300 000 EGF-R as determined by Scatchard analysis of [^{125}I]EGF binding experiments and [^{35}S]methionine labeling (Honegger *et al.*, 1987a).

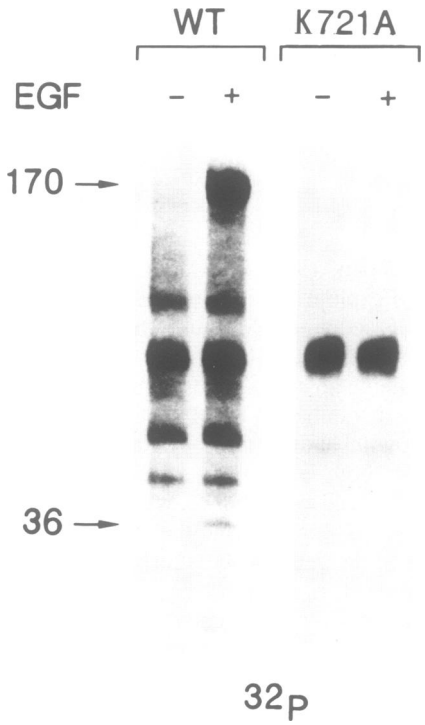

Fig. 1. EGF-dependent phosphorylation of EGF-R (170 kd) and a 36-kd membrane protein in plasma membrane preparations from NIH-3T3 cells expressing either wild-type (WT) or mutant EGF-R (K721A). Both cell lines expressing either wild-type or mutant EGF-R contain ~300 000 human EGF-Rs/cell (Honegger *et al.*, 1987a,b). For the phosphorylation assays, plasma membranes were pre-incubated for 15 min at room temperature with (+) or without (−) 5 μg/ml of EGF as described previously (Defize *et al.*, 1986). At the end of the phosphorylation reaction, the samples were analysed by SDS−PAGE and autoradiography.

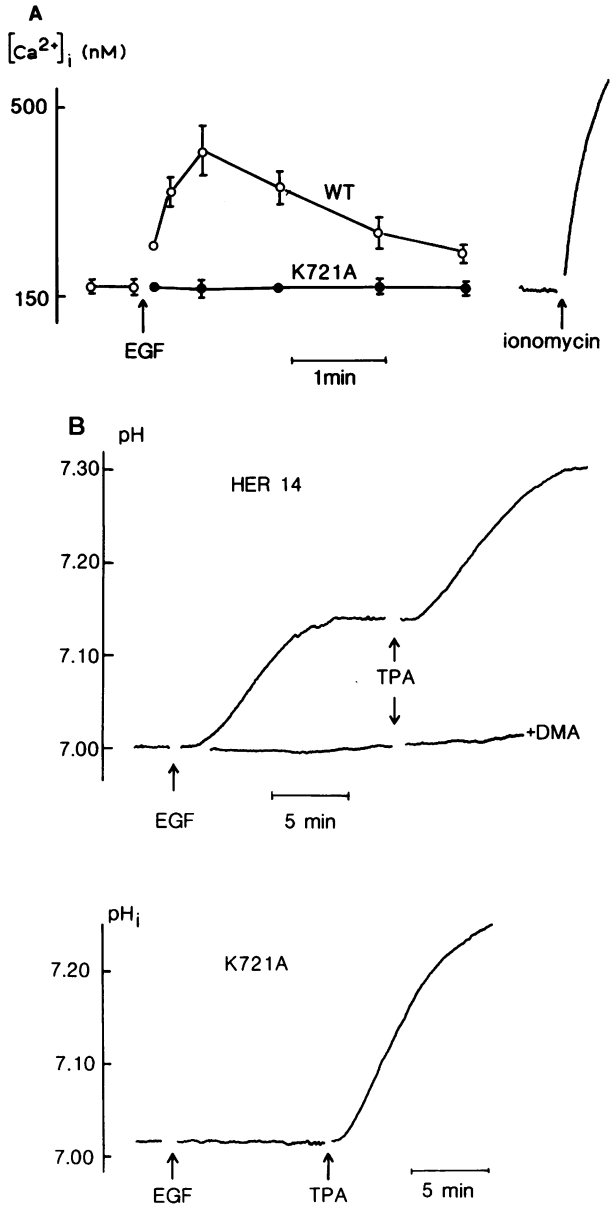

Fig. 2. Generation of ionic signals by EGF. (**A**) Changes in $[Ca^{2+}]_i$ after addition of 50 ng/ml EGF as measured by indo-1 fluorescence. Points represent mean (± SE) of five different experiments using either HER14 cells expressing wild-type (WT) EGF-R or K721A cells expressing TK-defective EGF-R. Both cell types respond to ionomycin (1 μg/ml) as illustrated. (**B**) Changes in pH_i after addition of EGF (50 ng/ml) or TPA (50 ng/ml) in the presence or absence of dimethylamiloride (DMA, 20 μM) as measured by fluorescence of BCECF in HER14 and K721A cells, respectively. Confluent cultures, attached to glass coverslips, were loaded with indo-1 or BCECF as described previously (Rothenberg *et al.*, 1983; Grynkiewicz *et al.*, 1985; Moolenaar *et al.*, 1986). Fluorescence monitoring, calibration procedures and other experimental details were as those described elsewhere (Rothenberg *et al.*, 1983; Moolenaar *et al.*, 1986).

Among the earliest detectable consequences of EGF-R interaction is a rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). As shown in Figure 2A, addition of EGF to HER14 cells expressing wild-type EGF-R elicits a rapid Ca^{2+} signal, as measured by the fluorescent Ca^{2+} indicator indo-1 (Grynkiewicz *et al.*, 1985). As in other EGF-responsive cells (Moolenaar *et al.*, 1986; Tilly *et al.*, 1987; Chen *et al.*, 1987), the $[Ca^{2+}]_i$ rise induced by EGF in HER14 cells originates largely from Ca^{2+} influx across the plasma membrane since removal of extracellular Ca^{2+} using excess EGTA abolishes the Ca^{2+} response to EGF by ~ 80% (data not shown). The mutant cells, however, fail to show any EGF-induced Ca^{2+} signal, whereas they do respond to the Ca^{2+}-ionophore ionomycin (Figure 2A). A similar conclusion was recently reached by Chen *et al.* (1987). However, the mutant EGF-R described in that study contains two mutations; Lys721 was replaced by a methionine residue and Thr654 was replaced by an alanine residue.

A well-known response to EGF and other growth factors is the activation of an otherwise quiescent amiloride-sensitive Na^+/H^+ exchanger in the plasma membrane (Moolenaar, 1986). This effect can be readily assessed by continuously monitoring amiloride-sensitive alterations in pH_i under bicarbonate-free conditions. A rapid, sustained rise in pH_i of ~0.20 unit is clearly detectable in EGF-treated NIH-3T3 cells expressing wild-type EGF-R (Figure 2B). This pH_i shift in response to EGF is abolished by dimethylamiloride (20 μM) and does not occur in Na^+-free media (data not shown), which indicates the involvement of Na^+/H^+ exchange. In contrast, cells expressing the TK-deficient receptor do not show a detectable pH_i shift (<0.01 unit) after EGF addition; however, the phorbol ester TPA evokes a marked pH_i response in cells expressing either wild-type or mutant EGF-R (Figure 2B).

Many cell surface receptors exert their effect, at least in

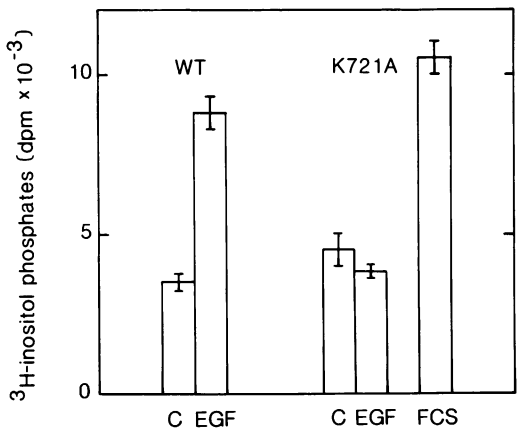

Fig. 3. Inositol phosphate formation induced by EGF. Confluent cultures were prelabeled with 4 μCi of [^{3}H]inositol/ml for 48 h. Analysis of total [^{3}H]inositol phosphates was described previously (Tilly *et al.*, 1987). FCS (7.5%, v/v) was used as a control stimulus to test responsiveness of the cultures, as shown here for K721A. HER14 cells gave a similar response to FCS (data not shown).

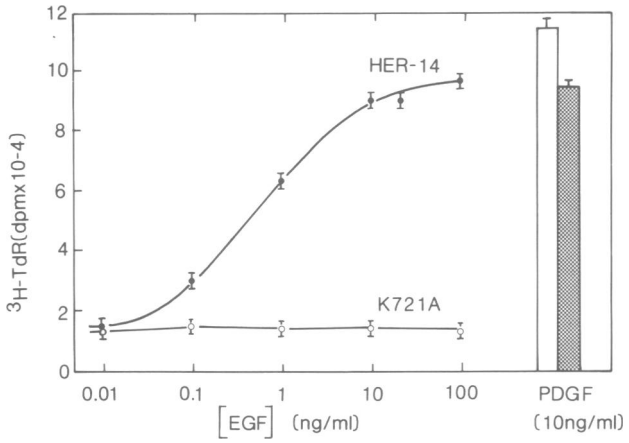

Fig. 4. EGF-induced DNA synthesis. Cells were plated in 24-well tissue culture dishes and grown to confluence in DMEM containing 7.5% FCS. Incubation was continued in DMEM containing 0.5% FCS for 24 h. Thereafter, cells were incubated in serum-free DMEM with the indicated concentrations of EGF or PDGF, and 6 h later the cultures were exposed to [^{3}H]thymidine (0.5 μCi/ml) for 18 h. TCA-precipitable material was quantified by liquid scintillation counting. The values are the mean (± SE) of triplicate experiments. Right part shows [^{3}H]thymidine incorporation induced by PDGF in HER14 cells (shaded bars) or K721A mutant cells (open bars).

part, through the inositol lipid-second messenger system, apparently with a specific G-protein as intermediate (Cockroft and Gompers, 1985; Berridge, 1987). Several recent studies report that the activated EGF-R in fibroblasts does not stimulate a measurable hydrolysis of phosphoinositides (Besterman *et al.*, 1986; L'Allemain and Pouysségur, 1986). In epidermoid carcinoma cells (A431) and in hepatocytes, on the other hand, EGF does evoke inositol lipid hydrolysis (Sawyer and Cohen, 1981; Tilly *et al.*, 1988; Johnson *et al.*, 1986; Pike and Eakes, 1987). HER14 cells expressing the wild-type receptor show a significant 2-fold increase of the level of total inositol phosphates in response to EGF (Figure 3). However, the TK-deficient EGF-R fails to mediate enhanced inositol phosphate formation, whereas the response to other stimuli, such as fetal calf serum (FCS) is unimpaired (Figure 3).

The ultimate biological response to growth factors is stimulation of DNA synthesis and cell division. EGF stimulates thymidine incorporation in HER14 cells with a dose–response similar to that in cells expressing endogenous EGF-R (Figure 4). Under the same conditions EGF is unable to stimulate DNA synthesis in cells expressing the mutant receptor, although these cells show a normal mitogenic response to FCS (Honegger *et al.*, 1987b) or platelet-derived growth factor (PDGF, Figure 4).

Taken together, our data indicate that abolition of the protein kinase activity of the EGF-R blocks the various receptor-linked signal pathways that ultimately lead to DNA synthesis. The simplest and most reasonable interpretation of the present findings is that the multiple post-receptor effects of EGF are a direct consequence of the activation of the receptor TK. It thus seems that EGF-induced phosphorylation of specific cellular substrates on tyrosine residues is a critical step in the pleiotropic response leading to DNA synthesis. The inability of the TK-defective receptor to stimulate inositol lipid hydrolysis is particularly intriguing, since it suggests that one or more of the key regulatory proteins in the phosphoinositide signalling cascade (e.g. G-proteins, phospholipase-C) may serve as a substrate for the EGF-R kinase. Identification of these substrates will be a major challenge for future studies.

Materials and methods

Transfections

NIH-3T3 cells (clone 2.2) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% FCS. 10 cm dishes of cells were transfected with 10–20 μg of construct DNA per dish using the calcium phosphate precipitation technique (Wigler *et al.*, 1979). Two days after transfection, the cells were split, seeded at a density of 100 000 cells/10 cm dish and put under neomycin resistance selection by adding 0.9 μg/ml of Geneticin (GIBCO) to the medium. Resistant clones were picked after 3 weeks, screened for [^{125}I]EGF binding and positive clones were tested for expression of human EGF-R by immunoprecipitation of [^{35}S]methionine-labeled receptor with human-specific antibodies. Full details about the preparation of the constructs and the characterization of the cell lines expressing them were recently described (Honegger *et al.*, 1987a,b).

Phosphorylation reaction

Plasma membranes prepared from the transfected cells were pre-incubated for 15 min at room temperature in the presence or absence of 5 μg/ml of EGF (Collaborative Research) as described previously (Defize *et al.*, 1986). The phosphorylation was started by adding buffer (pH 7.4) containing 2 mM $MnCl_2$, 10 μM ATP and 1 μCi of [γ-^{32}]ATP (Amersham) for 10 min at 4°C. The reaction was stopped by boiling in sample buffer and samples were analysed by SDS–polyacrylamide gels and by autoradiography.

pH_i measurements

Confluent culture cells, attached to glass cover-slips, were loaded with *bis*(carboxyethyl)carboxyfluorescein (BCECF) as previously described (Moolenaar *et al.*, 1986; Grynkiewicz *et al.*, 1985). Fluorescence monitoring, calibration procedures and other experimental details were similar to those described elsewhere (Moolenaar *et al.*, 1986).

$[Ca^{2+}]_i$ measurements

Changes in intracellular $[Ca^{2+}]_i$ after addition of 50 ng/ml EGF were measured by the indo-1 fluorescence method according to published procedures (Grynkiewicz *et al.*, 1985).

Inositol phosphate formation measurements

Confluent cultures were prelabeled to near isotopic equilibrium with 4 μCi of [^{3}H]inositol/ml for 48 h. The cells were stimulated with 50 ng/ml EGF in the presence of 10 mM LiCl for 30 min and the reactions were stopped by adding ice-cold TCA (10%, w/v). After centrifugation the supernatants were extracted with diethylether and neutralized extracts were processed for analysis of total [^{3}H]inositol phosphates by anion-exchange chromatography as described previously (Tilly *et al.*, 1987). FCS (7.5%, v/v) was used as a control stimulus to test responsiveness of the cultures.

DNA synthesis

Cells were plated in 24-well tissue culture dishes and grown to confluence in DMEM containing 7.5% FCS. Incubation was continued in DMEM containing 0.5% FCS for 24 h. Thereafter, cells were incubated in serum-free DMEM with EGF or PDGF, and 6 h later the cultures were exposed to [^{3}H]thymidine (0.5 μCi/ml) for 18 h. TCA-precipitable material was quantified by liquid scintillation counting.

Acknowledgements

We thank Dr Carl-Henrik Heldin for the gift of PDGF, L.Joosen, M.Simons and A.Fitzwater for typing the manuscripts. Supported in part by the Netherlands Cancer Foundation (Koningin Wilhelmina Funds).

References

Berridge,M.J. (1987) *Annu. Rev. Biochem.*, **56**, 159–193.
Besterman,J.M., Watson,S.P. and Cuatrecasas,P. (1986) *J. Biol. Chem.*, **261**, 723–727.
Carpenter,G. and Cohen,S. (1979) *Annu. Rev. Biochem.*, **48**, 193–216.
Chen,W.S., Lazar,C.S., Poenie,M., Tsien,R.Y., Gill,G.N. and Rosenfeld,M. (1987) *Nature*, **328**, 820–823.
Cockroft,S. and Gomperts,B.D. (1985) *Nature*, **314**, 534–536.
Defize,L.H.K., Moolenaar,W.H., van der Saag,P.T. and de Laat,S.W. (1986) *EMBO J.*, **5**, 1187–1192.
Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.
Honegger,A.M., Dull,T.J., Felder,S., Van-Obberghen,E., Bellot,F., Szapary,D., Schmidt,A., Ullrich,A. and Schlessinger,J. (1987a) *Cell*, **51**, 199–209.
Honegger,A.M., Szapary,D., Schmidt,A., Lyall,R., Van-Obberghen,E., Dull,T.J., Ullrich,A. and Schlessinger,J. (1987b) *Mol. Cell Biol.*, **7**, 4568–4571.
Hunter,T. and Cooper,J.A. (1985) *Annu. Rev. Biochem.*, **54**, 897–930.
Johnson,R.M., Connelly,P.A., Sisk,R.B., Pobiner,B.F., Hewlett,E.L. and Garrison,J.C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2032–2036.
L'Allemain,G. and Pouyssegur,J. (1986) *FEBS Lett.*, **197**, 344–348.
Moolenaar,W.H. (1986) *Annu. Rev. Physiol.*, **48**, 363–376.
Moolenaar,W.H., Aerts,R.J., Tertoolen,L.G.J. and de Laat,S.W. (1986) *J. Biol. Chem.*, **261**, 279–284.
Pike,L.J. and Eakes,A.T. (1987) *J. Biol. Chem.*, **262**, 1644–1651.
Rothenberg,P., Glaser,L., Schlessinger,P. and Cassel,D. (1983) *J. Biol. Chem.*, **258**, 12644–12653.
Sawyer,S.T. and Cohen,S. (1981) *Biochemistry*, **20**, 6280–6286.
Schlessinger,J. (1986) *J. Cell Biol.*, **103**, 2067–2072.
Tilly,B.C., van Paridon,P., Verlaan,I., Wirtz,K., de Laat,S.W. and Moolenaar,W.H. (1987) *Biochem. J.*, **244**, 129–135.
Tilly,B.C., van Paridon,P., Verlaan,I., de Laat,S.W. and Moolenaar,W.H. (1988) *Biochem. J.*, in press.
Wigler,M., Sweet,R., Sim,G.K., Wold,B., Pellicer,A., Lacy,E., Maniatis,T., Silverstein,S. and Axel,R. (1979) *Cell*, **16**, 777–785.

Received on November 16, 1987

Note added in proof

The present results are seemingly at variance with those of Defize *et al.* (1986) who reported that anti-EGF-R monoclonal antibodies could activate HE receptor kinase without producing EGF-like effects. However, subsequent experiments have shown that the phosphorylation pattern induced by these antibodies differs markedly from that induced by EGF. (Defize *et al.*, manuscript in preparation.)