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Dissociation of cellular responses to epidermal growth factor using anti-receptor monoclonal antibodies

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Three biologically active monoclonal antibodies against the human epidermal growth factor (EGF) receptor (2E9, 2D11 and 2G5) have been used to analyse the interrelationship between various cellular responses to EGF. Antibody 2E9 (IgG1) is directed against the protein core of the receptor, close to or at the EGF binding site, while 2D11 (IgG3) and 2G5 (IgG2a) recognize bloodgroup A-related carbohydrate determinants of the receptor. These antibodies have EGF-like effects in that they can activate the receptor tyrosine kinase both *in vitro* and *in vivo*. Cross-linking of the receptor-bound antibodies by a second antibody mimics EGF in inducing a rapid aggregation of receptors on the cell surface. However, all three antibodies fail to mimic EGF in raising cytoplasmic pH and free Ca^{2+} and do not stimulate DNA synthesis in quiescent fibroblasts, even after external cross-linking of the occupied receptors. It is concluded that (i) EGF–R tyrosine kinase activity as well as substrate specificity can be modulated by ligands other than EGF, even if they bind to sites distinct from the EGF binding domain; (ii) activation of the receptor tyrosine kinase, receptor clustering and induction of the ionic signals are causally unrelated events; and (iii) tyrosine kinase activation and receptor cross-linking are not sufficient for stimulation of DNA synthesis.

Key words: EGF receptor/ionic signals/monoclonal antibodies/receptor cross-linking/tyrosine kinase

Introduction

The binding of epidermal growth factor (EGF) to its plasma membrane receptor initiates a variety of biochemical and physiological changes in the target cell, usually leading to enhanced DNA synthesis and cell division (Carpenter and Cohen, 1979; Carpenter, 1984). Although much has been learned about the molecular structure of the EGF receptor, the mechanisms by which EGF induces its biological effects remain poorly understood. Much attention has been focused on the EGF-stimulated tyrosine-specific protein kinase activity which is intrinsic to the receptor (reviewed by Hunter and Cooper, 1985). This tyrosine kinase phosphorylates various substrates including the receptor itself (autophosphorylation), but whether activation of the receptor kinase is essential for a mitogenic response is not known.

Other immediate consequences of receptor activation include: (i) enhanced turnover of inositol phospholipids (Sawyer and Cohen, 1981); (ii) increases in cytoplasmic pH and free Ca^{2+} (Moolenaar *et al.*, 1983, 1984, 1986; Rothenberg *et al.*, 1983; Hesketh *et al.*, 1985); (iii) clustering and internalization of EGF–receptor complexes (Schlessinger *et al.*, 1978); and (iv) changes in cell morphology (Chinkers *et al.*, 1981). The relationship, if any, of these early EGF responses to activation of the tyrosine kinase remains to be determined.

For cause-and-effect analysis in the cascade of receptor-mediated events, the availability of agonists that activate the receptor-linked signal pathways only partially would be of great help. Monoclonal antibodies to the EGF receptor which can act as agonists (Schreiber *et al.*, 1981, 1983; Fernandez-Pol, 1985) or which can modify EGF binding (Kawamoto *et al.*, 1983) have previously been described. Other monoclonals do not display EGF-like effects but are useful for purification of the receptor (Parker *et al.*, 1984). The majority of the anti-receptor antibodies appears to be directed against bloodgroup A-related carbohydrate determinants carried by the receptor (Childs *et al.*, 1984). Three of these anti-carbohydrate monoclonals have been reported to mimic some of the effects of EGF (Schreiber *et al.*, 1983; Gregoriou and Rees, 1984; Fernandez-Pol, 1985). Of the antibodies which bind to undefined region(s) of the receptor, one has been claimed to mimic EGF action in all respects (Schreiber *et al.*, 1981).

We have examined the biological activities of three novel monoclonal antibodies against the human EGF receptor. The present study reports that these antibodies behave as partial agonists in that they are capable of stimulating the receptor tyrosine kinase activity both in membrane preparations and in intact cells.

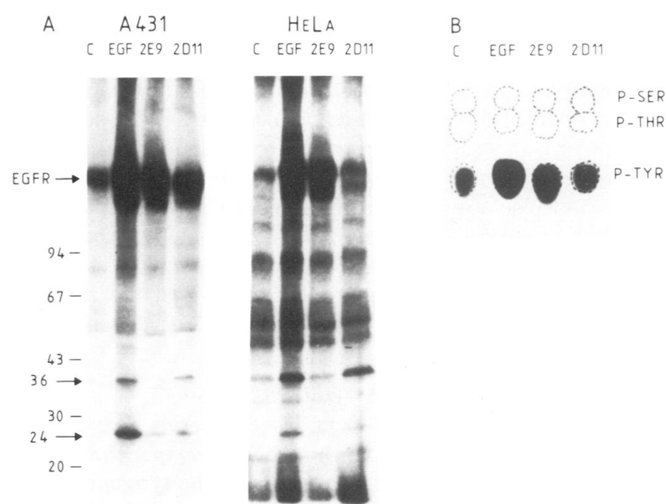

Fig. 1. (**A**) Autoradiograms of phosphorylated membrane proteins. Plasma membrane preparations of A431 (20 μg of protein) or HeLa cells (60 μg of protein) were incubated for 10 min at room temperature with the indicated ligands at a concentration of 1 μM. After this period, ^{32}P assay mix (see Materials and methods) was added and the mixture was incubated for an additional 10 min at 0°C. The kinase reaction was stopped by boiling in SDS–PAGE sample buffer and the samples were analyzed on linear 5–15% polyacrylamide gels. (**B**) Phosphoamino acid analysis of receptor bands cut out from the A431 gel shown in **panel A**. Receptor protein was hydrolysed in 6 M HCl after elution from the gel slices and subjected to high-voltage paper electrophoresis. The dotted circles represent ninhydrin-stained spots of phosphoamino acid markers on the paper. C represents control (non-immune mouse IgG, 1 μM.)

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Intriguingly, however, these antibodies fail to induce other EGF-like effects, including stimulation of DNA synthesis, even under conditions in which heavy macroclustering of receptors is observed. These, and several other, findings permit some new conclusions about EGF receptor functioning.

Results

Monoclonal antibodies against the human EGF receptor

We have produced and characterized three monoclonal antibodies against the EGF receptor from human A-431 epidermoid carcinoma cells. A complete description of their production and binding characteristics will be given elsewhere (Defize *et al.*, in preparation).

Briefly, antibody 2E9-IgG1 appears to recognize an antigenic determinant in the protein core of the receptor close to or at the EGF binding domain, since it can precipitate the non-glycosylated receptor and competes with EGF for receptor binding. Both EGF and 2E9 have $\sim 2 \times 10^6$ binding sites per A-431 cell. Antibodies 2D11-IgG3 and 2G5-IgG2a recognize different bloodgroup A-related carbohydrate structures on the receptor and do not detectably affect EGF binding. All three antibodies are human specific and react with a variety of human EGF receptor-bearing cells (Defize *et al.*, in preparation).

Monoclonal antibodies stimulate tyrosine-specific protein phosphorylation

To test for the possible EGF-like effects of the antibodies we first examined their ability to activate the receptor tyrosine kinase in plasma membranes prepared from A-431 and HeLa cells. Figure 1A shows an autoradiogram of phosphorylated membrane proteins in these membranes. It is seen that EGF markedly stimulates autophosphorylation of the 170-kd receptor as well as phosphorylation of two endogenous proteins with mol. wts of 36 and 24 kd respectively. Antibodies 2E9 and 2D11 mimic EGF in stimulating receptor autophosphorylation and, to a lesser extent, phosphorylation of the 24-kd protein; furthermore, 2D11, but not 2E9, causes phosphorylation of the 36-kd protein. In contrast, antibody 2G5 has no effect on membrane phosphorylation (not shown). In a separate series of experiments we used a total cell lysate of normal human fibroblasts (HF cells) as receptor source and also found a strong stimulation of receptor autophosphorylation by antibodies 2E9 and 2D11 (not shown).

The EGF-stimulated autophosphorylation activity is known to be specific for tyrosine. Figure 1B shows that the antibody-stimulated receptor phosphorylation is also exclusively on tyrosine residues.

Phosphorylation of tyrosine-containing peptide

Several tyrosine-containing peptides can serve as exogenous substrates for the EGF receptor kinase (Pike *et al.*, 1982; Wong and Goldberg, 1983). We have examined the tyrosine-specific phosphorylation of angiotensin I by A431 plasma membranes treated with increasing concentrations of EGF or antibody. Figure 2 shows that 2E9, like EGF, stimulates the phosphorylation of angiotensin I, whereas antibody 2D11 does not. In this assay we have also used monovalent 2E9 Fab fragments to test whether antibody bivalency is essential for activating the receptor kinase. As depicted in Figure 2 these fragments also are capable of stimulating the phosphorylation of angiotensin I, although to a lesser extent than 2E9-IgG, which is probably attributable to marked differences in binding affinity (unpublished data).

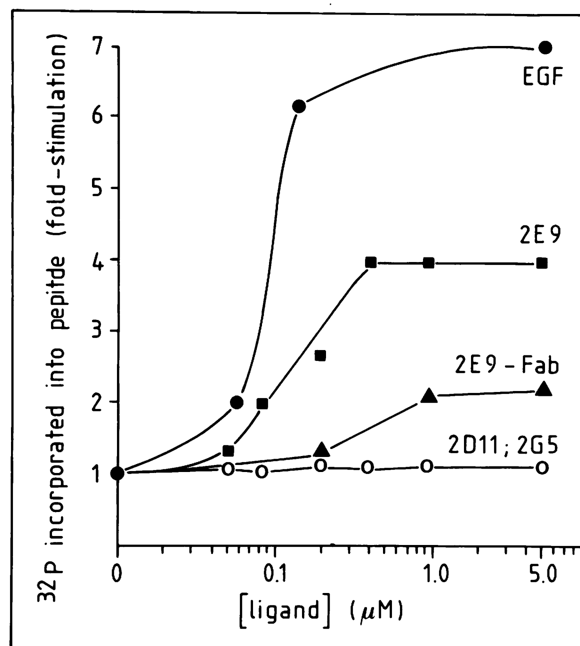


Fig. 2. Phosphorylation of angiotensin I by A431 plasma membranes. Membranes (20 μg of protein) were pre-incubated with the indicated amounts of ligand, followed by addition of ^{32}P assay mix (see Materials and methods) containing angiotensin I (2 mM) and the mixture was incubated for 3 min at 30°C. The reaction was stopped with TCA. Phosphorylated peptide was collected on Whatman P81 filters.

Kinase activation in intact cells

To test for the ability of the antibodies to stimulate the receptor kinase in intact cells, we metabolically labelled A431 cells with [^{32}P]orthophosphate for at least 12 h. Subsequently EGF or antibody was added for 60 min. EGF−R was immunoprecipitated from the solubilized cells using a polyclonal antiserum (RK-2; Kris *et al.*, 1985) directed against a cytoplasmic domain of the receptor, to ensure that equal amounts of receptor were precipitated from the different preparations. Figure 3A shows that binding of antibodies 2E9 and 2D11 to intact A431 cells indeed induces phosphorylation of the 170-kd receptor. Surprisingly antibody 2G5, which has no effect on isolated membranes (see above), also stimulates receptor phosphorylation. Phosphoamino acid analysis (Figure 3B) shows that antibody-stimulated phosphorylation, as with EGF, occurs not only on tyrosine but also on threonine and serine residues. The latter phosphorylations are thought to be mediated by kinases that are not intrinsic to the receptor (e.g. protein kinase C) (Hunter and Cooper, 1985).

Taken together, the above results show that antibodies 2E9, 2D11 and 2G5 are capable of mimicking EGF in activating the receptor tyrosine kinase activity. It appears, however, that the extent of activation depends on the experimental conditions used (cf. the action of 2G5 on membrane preparations versus intact cells) and that the substrate specificity of the tyrosine kinase is dependent on the nature of the ligand (cf. the phosphorylation of endogenous proteins versus that of angiotensin I).

Induction of receptor clustering

Subsequent to EGF binding, receptors rapidly cluster into patches that eventually are internalized by the cells. This energy- and temperature-dependent process can be visualized by conventional (immuno-) fluorescence techniques (Schlessinger *et al.*, 1978; Schreiber *et al.*, 1983). Various anti-EGF receptor antibodies have been reported to induce receptor clustering (Schreiber *et al.*, 1983; Gregoriou and Rees, 1984; Fernandez-Pol, 1985). We have tested our antibodies for their ability to aggregate recep-

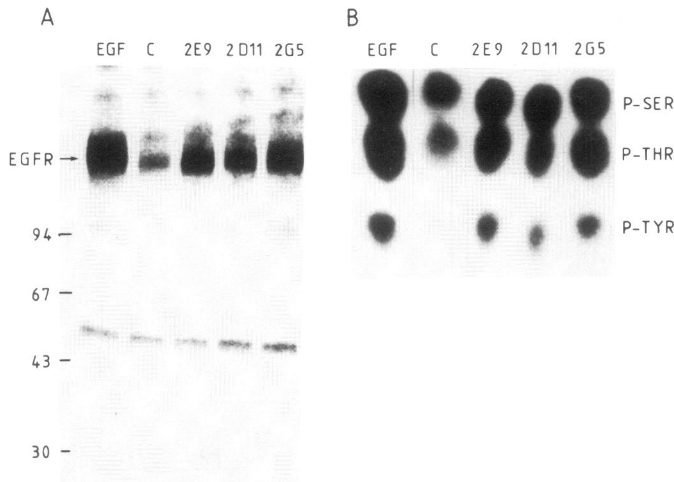


Fig. 3. (**A**) Autoradiograms of phosphorylated EGF receptor immunoprecipitated from lysates of ^{32}P-labelled A431 cells. Confluent A431 cultures were labelled with 0.5 mCi/ml [^{32}P]orthophosphate for 12–16 h at 37°C in phosphate-free DMEM. The indicated ligands were added to the same medium for an additional hour. Subsequently, cells were scraped off the dish and lysed in buffer containing EDTA. EGF–R was precipitated using a polyclonal anti-receptor peptide antibody coupled to protein A–Sepharose beads (see Materials and methods). Precipitates were boiled in Laemmli sample buffer and analyzed on a 5–15% linear polyacrylamide gel. Concentrations of the ligands EGF; 8 nM; 2E9, 30 nM; 2D11 and 2G5, 300 nM. (**B**) Phosphoamino acid analysis of individual receptor bands cut out from the gel shown in **panel A**. Analysis as described in Materials and methods and Figure 1B legend. C represents control (non-immune mouse IgG, 300 nm).

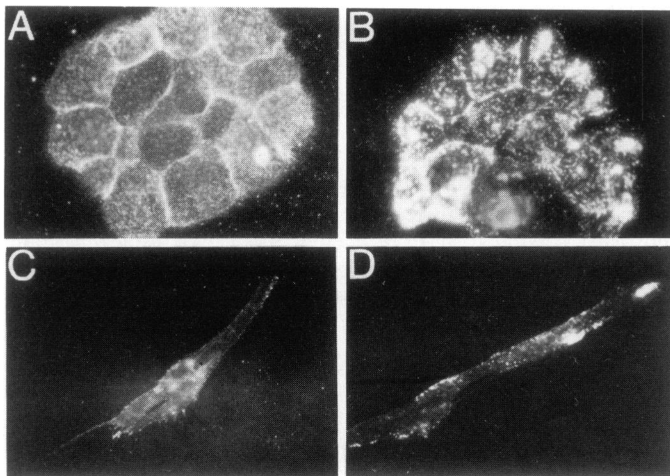
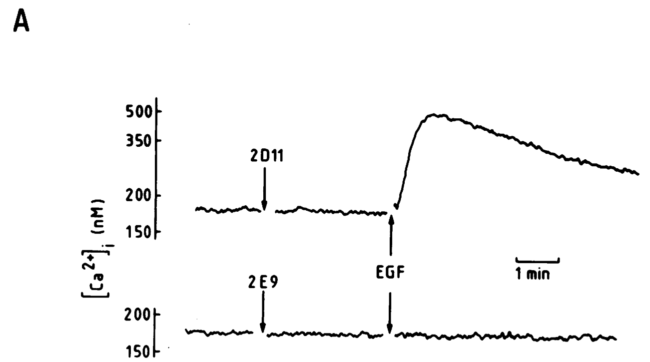


Fig. 4. Distribution of 2E9-IgG on A431 (**A** and **B**) and HF cells (**C** and **D**). Cells on glass coverslips were incubated with 2E9 (30 μg/ml) for 60 min at 37°C, washed and incubated with fluorescein-conjugated rabbit anti-mouse antibodies (80 μg/ml) either for 2 h at 0°C (**A** and **C**) or from 1 h at 0°C followed by warming to 37°C for 30 min to induce clustering (**B** and **D**). Cooling and warming of the cells before addition of the second antibody did not influence the distribution of fluorescence (magnification × 400).

tors in A431 and HF cells. None of the antibodies, which are bivalent, induce detectable receptor redistribution at 37°C, as shown for 2E9 in Figure 4A and C using fluorescein-labeled anti-mouse IgG at 0°C. However, when the monoclonals already bound to the receptor are cross-linked by anti-mouse IgG at 37°C, clustering rapidly occurs and is essentially complete after 30–60 min (Figure 4B and D). It thus seems that external



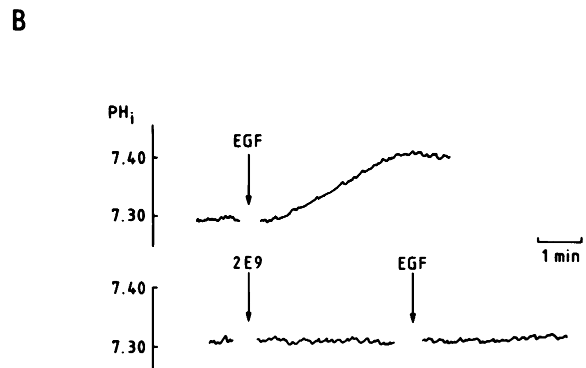


Fig. 5. Measurements of $[Ca^{2+}]_i$ and pH_i in A431 cells. Cells were grown to near confluency on glass coverslips and loaded with either quin-2 (**panel A**) or BCECF (**panel B**). Fluorescence from the cells was continuously recorded as described previously (Moolenaar *et al.*, 1983, 1986). Concentrations used: 2E9 and 2D11, 1–30 μg/ml; EGF, 100 ng/ml.

cross-linking of receptors is sufficient for visible clustering to occur.

Lack of effect on cytoplasmic pH and free Ca^{2+}

In addition to stimulating tyrosine-specific protein kinase activity, EGF elicits a rapid but transient rise in free Ca^{2+} ($[Ca^{2+}]_i$) and activates the plasma membrane $Na^+ - H^+$ exchanger to raise cytoplasmic pH (pH_i) in its target cells (Moolenaar *et al.*, 1983, 1984, 1986; Rothenberg *et al.*, 1984; Hesketh *et al.*, 1985). When added at concentrations that stimulate tyrosine kinase activity, all three anti-receptor monoclonals completely failed to mimic EGF in raising $[Ca^{2+}]_i$ and pH_i in A431 cells (see Figure 5 for typical examples). Even after addition of a cross-linking anti-mouse IgG to induce receptor clustering (see above), there is no detectable increase in $[Ca^{2+}]_i$ and pH_i (not shown).

Figure 5 (A and B) further illustrates the antagonistic effect of monoclonal 2E9, which competes with EGF for receptor binding, on the EGF-induced ionic responses. As expected, the anti-carbohydrate monoclonal 2D11 does not prevent an ionic response to EGF (Figure 5A). These results support the concept that activation of the protein tyrosine kinase and induction of the ionic signals by EGF are causally unrelated events.

Morphological changes in A431 cells

EGF has dramatic effects on the morphology of A431 cells. Within minutes of exposure to EGF extensive membrane ruffling occurs which is followed by cell rounding (Chinkers *et al.*, 1981). The latter effect is particularly prominent in low Ca^{2+} media, but is also observed under normal conditions at low cell density (unpublished observations). The anti-carbohydrate

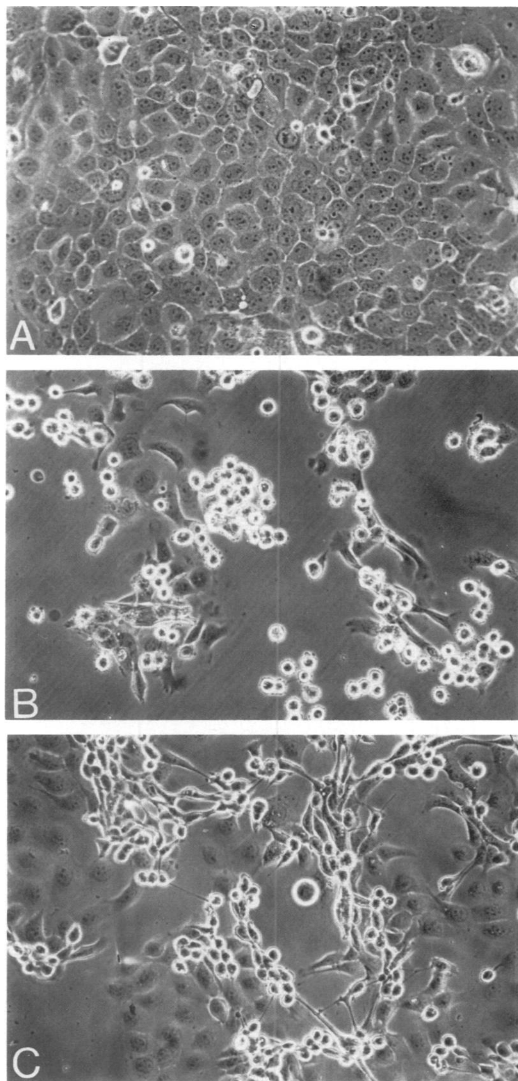


Fig. 6. Phase-contrast images of A431 cells incubated with EGF or antibody. A431 cells were grown to near confluency (100 000 cells/cm^2). After washing the cells, antibody 2E9 (30 μg/ml) (**panel A**), EGF (100 ng/ml) (**panel B**) or antibody 2D11 (30 μg/ml) (**panel C**) were added. Cells were photographed after an incubation period of 10 min (37°C). Magnification × 200.

monoclonal 2D11 exerts an EGF-like effect in causing rapid rounding of A431 cells, whereas 2E9 and 2G5 have no visible effect on cell morphology (Figure 6 and unpublished observations). The kinetics of cell rounding induced by 2D11 are identical with those induced by EGF. In both cases cell rounding is complete within 10–20 min at 37°C in the presence of 1.8 mM Ca^{2+}.

Cross-linking of receptor-bound 2E9 or 2G5 by a second antibody to induce receptor aggregation (see above) does not restore the shape changes (not shown). This suggests that receptor clustering and cell rounding in A431 cells are mediated via separate signal pathways.

Antibodies fail to stimulate DNA synthesis

Of critical importance is the question as to whether the antibodies, in addition to their capacity to activate the receptor tyrosine kinase, are able to stimulate DNA synthesis in EGF-responsive human cells. Figure 7 shows that none of the monoclonals are capable of stimulating [^{3}H]thymidine incorporation in quiescent HF cells. We tested various concentrations of the antibodies

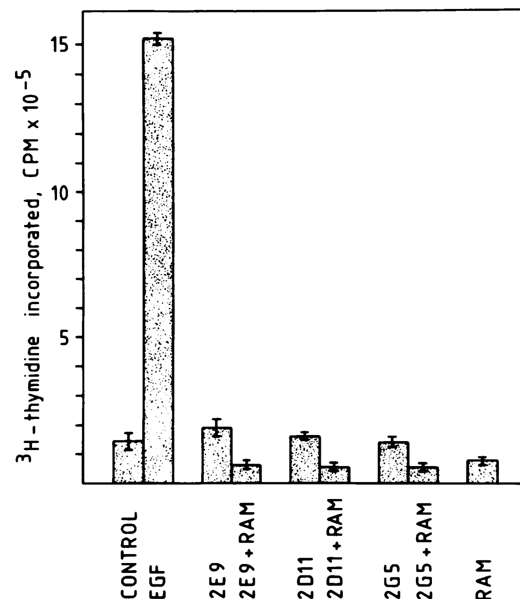


Fig. 7. [^{3}H]Thymidine incorporation in quiescent fibroblasts. Serum-deprived HF cells were incubated with the indicated ligands for 18 h prior to a 6-h pulse with [^{3}H]thymidine and determination of acid-precipitable radioactivity (see Materials and methods). Some dishes received rabbit anti-mouse antibodies (RAM) (80 μg/ml) 1 h after addition of the monoclonals to induce receptor clustering (cf. Figure 4). Concentrations used: EGF, 15 ng/ml; 2E9, 2D11 and 2G5, 30 μg/ml.

(1–30 μg/ml) alone or in combination with increasing amounts of a second, cross-linking antibody (1–80 μg/ml) but were unable to detect any stimulatory effect on DNA synthesis (Figure 7). This result is at variance with that of Schreiber *et al.* (1983), who claimed that cross-linking of EGF receptors is a sufficient signal for the initiation of DNA synthesis.

Discussion

Our results demonstrate that monoclonal antibodies against the EGF receptor can induce some of the early biochemical events normally produced by EGF itself without evoking a mitogenic response. They thus can be regarded as partial receptor agonists that may serve as useful tools to dissect the various signal pathways in the action of EGF. Table I summarizes the major properties and biological activities of the three anti-receptor monoclonals used. All three monoclonal IgGs stimulate the tyrosine-specific autophosphorylation of the EGF receptor in intact cells, an effect previously observed only for a multivalent anti-receptor antibody of the IgM class (Fernandez-Pol, 1985). Interestingly, the antibodies have different effects on the specificity of the tyrosine kinase for other substrates (Table I). This suggests that activation of the tyrosine kinase by an external ligand is a relatively complex event rather than an all-or-none phenomenon. Of course, it remains to be determined whether or not EGF and the antibodies induce autophosphorylation of the receptor at identical sites, since the nature of the phosphorylation site(s) could conceivably influence the enzyme's substrate specificity. It is clear from Figure 3B that binding of the antibodies to intact cells, like EGF, causes additional phosphorylation of the receptor on threonine and serine residues. The latter phosphorylations have been observed by others (Hunter and Cooper, 1981; Cochet *et al.*, 1984) and are probably due to the action of kinases that are not intrinsic to the receptor protein kinases (Bertics and Gill, 1985). One possible candidate is the calcium- and phospholipid-

Table I. Summary of the binding properties and biological actions of the antibodies

	EGF	2E9	2D11	2G5
(Sub)class		IgG1	IgG3	IgG2a
Carbohydrate reactive	−	−	+	+
Binding competition with [^{125}I]EGF	+	+	−	−
Induction of receptor tyrosine kinase activity:				
(a) Autophosphorylation of receptor in membrane preparation	+	+	+	−
(b) Phosphorylation of 36-kd endogenous substrate	+	−	+	−
(c) Phosphorylation of 24-kd endogenous substrate	+	+	+	−
(d) Autophosphorylation of receptor in intact cells	+	+	+	+
(3) Phosphorylation of angiotensin I	+	+	−	−
Cytoplasmic alkalinization	+	−	−	−
Rise in cytoplasmic Ca^{2+} concentration	+	−	−	−
Rapid morphological changes	+	−	+	−
EGF−R clustering	+	+[a]	+[a]	+[a]
[^{3}H]Thymidine incorporation	+	−	−	−

[a]Only in combination with a second, cross-linking antibody.

dependent protein kinase C, which is able to phosphorylate the receptor on a threonine residue close to the plasma membrane (Hunter *et al.*, 1984; Davis and Czech, 1985). However, since the antibodies do not stimulate phosphoinositide breakdown (B.C.Tilly *et al.*, unpublished observations), we feel it is unlikely that kinase C is involved.

Whatever the nature of the receptor (auto)phosphorylation sites, our results obtained with the anti-carbohydrate antibodies (2D11 and 2G5) support the view that ligands can activate the receptor kinase through domains distinct from the EGF binding site (cf. Fernandez-Pol, 1985), and that such ligands, like EGF, can render the receptor susceptible to phosphorylation by other intracellular kinases.

Antibody 2D11, but not 2E9 or 2G5, mimics EGF in inducing the rapid rounding of A431 cells. There appears to be a correlation between the 2D11- and EGF-induced cell rounding and the phosphorylation of the 36-kd membrane-associated protein (Table I). It is premature to speculate about the functional role of the 36-kd substrate, if any, in mediating shape changes in A431 cells, but we note that a 36-kd tyrosine kinase substrate is thought to be involved in cytoskeleton−plasma membrane interactions (Lehto *et al.*, 1983).

None of the antibodies is capable of raising pH_i and $[Ca^{2+}]_i$ as does EGF. If one accepts the view that these ionic signals may be generated through the receptor-linked activation of phospholipase C (Moolenaar *et al.*, 1984, 1986; Berridge and Irvine, 1984), this would imply that triggering of the EGF receptor tyrosine kinase is not sufficient to stimulate inositol lipid breakdown. Preliminary experiments indeed confirm that the antibodies, unlike EGF, fail to raise inositol phosphate levels in A431 cells (B.C.Tilly *et al.*, unpublished data).

One possibility to explain the failure of the antibodies to evoke a full biological response, including enhanced DNA synthesis, involves microaggregation of the receptors, which has been hypothesized to be sufficient for the expression of a complete EGF-like spectrum of action (Schreiber *et al.*, 1981, 1983; Schlessinger *et al.*, 1983). In an attempt to verify this hypothesis we have cross-linked the cell-bound antibodies by using a second antibody (anti-mouse IgG) to induce full biological activity. However, all of our attempts were negative. While the cross-linking procedure did induce visible (macro-) clustering of receptors (Figure 4), there was no detectable effect on the ionic parameters or DNA synthesis. We recognize that external cross-linking of receptor-bound antibodies is a difficult procedure that requires a subtle stoichiometry between the various ligands. We therefore feel that the microaggregation hypothesis cannot be completely rejected simply based on the present results, since they could reflect differences in experimental approach. However, our results do allow the conclusion that the formation of visible 'macro-clusters' of EGF receptors on the cell surface (Figure 4) is neither sufficient for the generation of ionic signals nor for the stimulation of DNA synthesis.

In conclusion, our results demonstrate that the EGF receptor kinase can be triggered to catalyze its autophosphorylation as well as the phosphorylation of some other substrates, both natural and synthetic, without inducing receptor redistribution, intracellular ionic changes or a mitogenic response. We cannot exclude the possibility that the absence of the latter responses in the case of the antibodies are due to the reported differences in substrate specificity of the kinase, but our results support the view that the initiation of DNA synthesis by EGF is not mediated through a single biochemical pathway solely involving stimulation of tyrosine-specific protein kinase activity. Additional intracellular signals, perhaps including the rises in $[Ca^{2+}]_i$ and pH_i, are apparently indispensable for the stimulation of DNA synthesis by EGF.

Materials and methods

Cell culture

Cell lines (A431 and HeLa) and secondary human foreskin fibroblasts (HF) were routinely grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 7.5% fetal calf serum (FCS; Flow Laboratories) and buffered with 44 mM $NaHCO_3$ under a 7% CO_2 atmosphere.

Monoclonal antibodies

EGF receptor-reactive monoclonal antibodies 2E9 (IgG1), 2D11 (IgG3) and 2G5 (IgG2a) were obtained by immunization of BALB/c mice with a plasma membrane preparation of A431 cells and fusion of the spleen cells with myeloma line SP2/0, as will be described elsewhere (Defize *et al.*, in preparation). For purification, 2 ml of antibody-containing ascites fluid was adjusted to pH 8.1 and applied on a 5 ml protein A−Sepharose column (Pharmacia). Bound antibody was eluted at pH 3.0 with 0.1 M acetic acid. Samples were collected in tubes containing a few drops of 1 M Tris−HCl pH 8.0 to restore pH. The eluate was dialyzed against phosphate-buffered saline (PBS) and concentrated in a collodion bag (Sartorius) under reduced pressure. Purified antibodies were kept in small aliquots at −70°C and were stable for at least 6 months. Monovalent Fab fragments were prepared by using immobilized papain (Pierce) according to the manufacturer's instructions.

Membrane preparation

Plasma membranes were prepared according to the method described by Thom *et al.* (1977). For endogenous phosphorylation assays, the membrane preparations were diluted wth PBS containing Ca^{2+} and Mg^{2+}.

Phosphorylation assays

For endogenous phosphorylation assays, receptor preparations (membranes, precipitates) were pre-incubated for 15 min at room temperature with ligand. The phosphorylation reaction was started by adding ^{32}P 'assay mix' containing 20 mM Hepes pH 7.4, 2 mM $MnCl_2$, 10 mM *p*-nitrophenylphosphate (PNPP), 40 μM Na_3VO_4, 0.01% bovine serum albumin (BSA), 15 μM ATP and 1 μCi [γ-32P]ATP and 0.2% Nonidet P-40 (NP-40) in a final volume of 60 μl. The mixture was incubated for a further 10 min on ice. The reaction was stopped by boiling in Laemmli sample buffer and samples were applied to a 5–15% linear gradient gel. Dried gels were autoradiographed using Kodak XAR-5 film with Kodak X-Omatic regular intensifying screens.

For exogenous phosphorylation assay using angiotensin as a substrate, 20 μg of plasma membrane protein was pre-incubated with ligand for 10 min at room temperature. Subsequently, ^{32}P assay mix (see above) was added, containing 2 mM angiotensin I (Sigma) in a final volume of 40 μl. The mixture was incubated for 3 min at 30°C. The reaction was stopped by adding 50 μl of 5% trichloroacetic acid (TCA) and 20 μl of 2.5% (w/v) BSA. After centrifugation (4 min, 10 000 *g*) the supernatant was applied to phosphocellulose paper (Whatman P81). Papers were washed six times in 7.5 mM phosphoric acid. Filters were dried and radioactivity was measured by liquid scintillation counting. Correction for aspecific phosphorylation was made by omitting the peptide from the assay mix.

^{32}P labelling of intact A431 cells

A431 cells were grown to confluence in 10 cm^2 dishes. For labelling with [$^{32}P_i$]-orthophosphate, cells were incubated in 1 ml of phosphate-free Earle's modified Eagle's medium (EMEM; Gibco), supplemented with 1% FCS and containing 0.5 mCi of [$^{32}P_i$]orthophosphate (Amersham). Cells were incubated for 12–16 h at 37°C overnight.

Immunoprecipitation of membrane and metabolically labelled cell lysates

Plasma membrane preparations were solubilized in 20 mM Hepes (pH 7.4)/10% glycerol/1.5% Triton X-100. Insoluble material was removed by centrifugation at 100 000 *g* for 60 min at 4°C. ^{32}P-Labelled cells were scraped in isotonic buffer containing 20 mM Tris pH 8.0/2 mM EDTA/100 mM NaCl/10 mM Na_2HPO_4/0.1% BSA/1 mM Na_3VO_4/50 mM NaF/5 mM PNPP/1.5% NP-40/1 mM phenylmethylsulphonyl fluoride (PMSF) and 100 KIU/ml of aprotinin. The cell lysates were centrifuged at 12 000 *g* for 15 min at 4°C in an Eppendorf microfuge. Solubilized fractions were incubated with antibody for 60 min at 4°C in Eppendorf tubes that were rotated end over end, and then 50 μl of a 1:1 pre-washed suspension of protein A–Sepharose beads (Pharmacia) coupled to a polyclonal anti-mouse Ig serum (Nordic, Tilburg, The Netherlands) was added. After a further incubation for 30 min at 4°C the beads were spun down and washed twice with 1.5 ml of 0.5 M NaCl in Tris-HCl (pH 7.4) and three times with 0.15 M NaCl in Tris-HCl (pH 7.4) buffer. For subsequent SDS gel electrophoresis on linear 5–15% polyacrylamide gels, the beads were boiled in 50 μl Laemmli sample buffer for 5 min.

Phosphoamino acid analysis

Phosphorylated EGF receptor bands were excised from dried gels, allowed to swell in a 100 mM $NaHCO_3$ solution and incubated twice for 24 h at 37°C in 100 μg/ml trypsin while shaking. After centrifugation, supernatant was dried in a speed-vac concentrator (Savant). The dried samples were hydrolyzed in 6 N HCl (0.4 ml) for 120 min at 110°C under reduced pressure. Hydrolysates were subsequently dried and resuspended in 10 μl H_2O containing phosphoserine, phosphothreonine and phosphotyrosine as markers. The samples were spotted onto Whatmann filter paper and subjected to high voltage paper electrophoresis (pH 3.5, 1500 V, 1.5 h) in 5% acetic acid, 5 mM EDTA adjusted to pH 3.5 with pyridine. Markers were visualized by ninhydrin and radioactivity was localized by autoradiography.

[^3H]Thymidine incorporation

HF cells, taken from a confluent culture, were plated (1:2 split ratio) in 24-well tissue culture plates. Cells were grown to confluence (3 days) in serum-containing DMEM. Incubation was continued in serum-free DMEM for an additional 3 days. Thereafter EGF or antibodies were added to the serum-free medium and 18 h later cells received a 6-h pulse of [^3H]thymidine (0.5 μCi/ml). Subsequently, TCA-precipitable material was quantified in a liquid scintillation counter.

$[Ca^{2+}]_i$ and pH_i

$[Ca^{2+}]_i$ and pH_i measurements were carried out on A431 cells attached to glass coverslips in a fluorescence spectrometer (Perkin-Elmer 3000), using the Ca- and pH-sensitive probe quin-2 and bis(carboxyethyl)carboxy-fluorescein (BCECF) respectively. For experimental details see Moolenaar *et al.* (1983, 1984, 1986).

Acknowledgements

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