A simple luciferase assay for signal transduction activity detection of epidermal growth factor displayed on phage

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ABSTRACT
Studies on receptor–ligand interactions are important for the design of agonists or antagonists of natural ligands. We developed a luciferase reporter assay to screen epidermal growth factor receptor (EGFR) binding molecules rapidly for their ability to stimulate or inhibit signal transduction. Human EGF displayed on fd filamentous phage presented an activity similar to soluble EGF when tested for binding to the EGFR, for induction of cell cycle progression or in the luciferase assay. Two libraries of human EGF variants displayed on phage were constructed in which the aspartic acid residue at position 46 or the arginine residue at position 41 were randomised. EGF mutants displayed on phage were screened in parallel for binding to the EGFR using an ELISA assay and for transducing activity using the luciferase assay. Regarding the 46 position, most of the mutants retained the ability to bind the EGFR and their transducing activity corresponded perfectly with their binding. For the more crucial 41 position, only the wild-type EGF was able to bind the EGFR. Our approach allowed a simple determination of crucial positions and paved the way for identification of agonists with altered transduction activity.

INTRODUCTION
Human epidermal growth factor (EGF) is a 53 amino acid peptide which acts as a growth factor on a wide variety of cell types. Binding of the EGF to its membrane receptor results in the activation of intracellular signalling pathways which lead to essential biological functions such as cell growth and differentiation (1). EGF binding triggers EGF receptor (EGFR) dimerisation, followed by activation of the receptor’s intracellular kinase activity (2) and autophosphorylation of the C-terminal ends of the receptor dimer (1,3). The phosphorylated receptor adopts a conformation which is competent in phosphorylating cellular substrates (4). Most of these substrates are kinases involved in different signal transduction pathways (5). Following EGF binding, EGF/EGFR complexes are rapidly internalised and enter distinct cellular sorting pathways leading to either degradation or recycling to the cell surface (1).

Overexpression or deregulation of the EGFR is thought to play a critical role in several human tumors such as mammary and ovarian carcinoma. The autocrine stimulation of the overexpressed receptor is essential in achieving a transforming effect (6–8). Blocking antibodies, which prevent EGF binding or EGFR autophosphorylation, can inhibit tumor proliferation (9–11). Therefore, the development of novel antagonists has a great clinical potential.

So far, no structure of EGF in complex with its receptor has been determined. However, a model for the receptor binding site was proposed based on the high resolution NMR structure of the free EGF molecule (12), sequence comparison and mutational studies (13–17). It was found that several residues were necessary for EGF binding to its receptor: specifically Tyr13, Leu15, His16, Arg41, Gln43 and Leu47.

Phage display technology (18) has been extensively used to select specific peptide and protein ligands (19). Foreign DNA fragments are inserted into the genome of filamentous phages in fusion with coat protein genes. Through fusion with the minor coat protein g3p, of which three to five copies are displayed on the phage head surface (18), the genotype (the specific coding DNA sequence) and phenotype (the foreign protein exposed on the surface) are physically linked. This allows the selection and rescue of rare peptides or proteins with the desired ligand specificity by repeated rounds of affinity purification on immobilized antigens.

In this report, we displayed human EGF on an fd phage surface (phage-EGF), and tested its ability to bind to the EGFR and to induce DNA synthesis, which represents a specific cellular feature of EGFR-induction downstream on the signal transduction pathway. We described an assay for EGFR transduction activity utilising the luciferase gene under control of the c-fos gene enhancer SIE (v-sis inducible element) (20,21) as a reporter. The test was used to rapidly screen and compare the transduction activity of human EGF mutants displayed on the phage surface.

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MATERIAL AND METHODS

Display of EGF on phage

A synthetic human EGF (22) was amplified by PCR with the primers EGFback (5′-GGCGTGACGCCAGCAATTTCCAGCAGG-3′) and EGFfor (5′-AAGTGCGCCAGGGCCAGCCGAGTTCCCCACC-3′) and cloned into the phage fd fuse5 vector as above.

Construction of the EGF mutants libraries

Two complementary oligonucleotides (5′-GAATTCATTCGACCCGTGCTTCTACGATGGAATGC-3′) and (5′-AGGTTGATCCGGGGAGGCTACGGAATGC-3′), containing three copies of the c-fos serum inducible element (SIE) (20) were cloned into the phage fd fuse5 vector as above. Phages were produced as described in the literature for fd filamentous phages (24).

The same strategy was adopted to construct the arginine (R) 41 randomised library using the degenerate primer R41EGF for (5′-EGF for (5′-GGCGTGACGCCAGCAATTTCCAGCAGG-3′). M indicates a mixture of C and A; N represents any of the four nucleotides. The PCR fragments were cloned into the phage fd fuse5 vector as above. Phages were produced as described in the literature for fd filamentous phages (24).

Construction of the luciferase reporter vector

Two complementary oligonucleotides (5′-GATCCCCATTTCCGTAAATCCATTTCCCGTAAATC-3′) and (5′-AGCTTTAGGAAATGGTAGGCTATCCAGGAAATGC-3′), containing three copies of the c-fos serum inducible element (SIE) (20) were cloned into the BamHI and HindIII restriction sites of the pzLucTK vector (25) upstream of the luciferase cDNA yielding the vector pzLucTKSIE. The same cloning strategy was used to construct the control vector pKSSIE using a pBluescript II (KS)+ vector.

Cell culture

Monkey COS-7, human carcinoma A431 cells (expressing ~10⁶ EGFR per cell), NIH 3T3 cells, clone 2.2 cells (NIH 3T3 cells lacking endogenous murine EGFR) and its derivative clone 2.2 wt, expressing the human EGFR (~4 × 10⁵ per cell) (26), were grown in DMEM supplemented with 10% fetal calf serum (FCS).

Cell transfection

For transient transfection, COS-7 cells were cotransfected by lipofection (LipofectAMINE™, GIBCO) for 5 h, with 1 µg each of pRK5 EGF-R (27) and pzLucTKSIE (or pZLucTK as a negative control). Stably transfected 2.2 SIE fibroblasts were obtained by cotransfection of 2.2 wt cells using 6 µg of the luciferase vector pzLucTKSIE and 0.3 µg of the plasmid pTKhygro conferring hygromycin resistance (28). The day after transfection, cells were amplified in 100 mm Petri dishes and 50 µg/ml hygromycin (Boehringer Mannheim) was added. After 12–15 days of selection, clones were amplified and tested for luciferase activity.

Assay for DNA synthesis

Subconfluent NIH 3T3 cells were serum starved for 48 h and then growth stimulated for 18 h in the presence of 100 nM bromodeoxyuridine (BrDU). Cells were stimulated upon addition of either soluble EGF (25 ng/ml = 2.5 × 10¹⁰ EGF molecules/ml), phase-EGF (5 × 10¹³ phase displayed EGF molecules/ml) or control phase displaying Fab fragments (5 × 10¹² phase displayed Fab molecules/ml) randomly picked from naïve libraries (24). Cells were then fixed with 3% paraformaldehyde for 10 min at room temperature, washed with cold phosphate buffered saline (PBS) and permeabilised with cold acetone for 30 s. After rinsing in cold PBS, cells were incubated 10 min in 4N HCl and washed again with PBS. An anti-BrdU antibody (Dako C0747) and 10 µM Hoechst dye (Sigma B2883) was added followed by a FITC-conjugated anti-mouse antibody (Sigma F5387). Coverslips were mounted in mowiol and examined with a Zeiss Axiophot microscope using appropriate filters.

ELISA assay on cells

4 × 10⁵ cells were fixed on 96-well plates treated with poly-L-lysine (1 mg/ml in PBS) for 15 min at room temperature. After blocking with 2% dry milk in PBS, phage supernatants diluted in 2% dry milk in PBS were incubated for 1 h and rinsed extensively with PBS containing 0.1% Tween 20. An anti-M13 phage antibody conjugated with horse radish peroxidase (HRP) (Pharmacia) was then incubated to detect bound phages. The ELISA was developed with soluble peroxidase substrate (Sigma T405).

Luciferase assay

For assays in 6-well culture plates, 10⁶ cells in 2 ml medium were serum starved for 24 h and stimulated for 3 h with EGF or phase-EGF. For assays in 96-well culture plates, 4 × 10⁵ cells in 100 µl of medium were starved 12–24 h before stimulation. Cells were rinsed with cold PBS and lysed for 10 min in 250 µl (6-well plate) or 50 µl (96-well plate) 15 mM phosphate buffer, pH 7, containing 2 mM ATP, 1 mM DTT, 8 mM MgCl₂, 0.5% Triton X-100. Luciferase activity was assayed using luciferase substrate buffer (0.1 mM EDTA, 20 mM tricine, 1 mM MgCl₂, 2.64 mM MgSO₄, 33.3 mM DTT, 270 µM acetyl-coenzyme A (Boehringer 101907) and 470 µM o-luciferine (Boehringer 411400). Competition experiments were performed as described above. Soluble EGF or phase-EGF supernatant diluted in medium culture were mixed with either blocking antibody (IgG 528, Santa Cruz sc120) (31) or non-blocking antibody (clone 29.1, Sigma E-2760) before cell stimulation.

RESULTS AND DISCUSSION

Phage display of human EGF

Human EGF (22) was displayed on the surface of fd phage FUSE5 through fusion to the N-terminus of the minor coat protein g3p. Resulting phage-EGF particles readily infected Escherichia coli indicating that the g3p function was restored. Phage concentration, determined spectrophotometrically or by DNA extraction (29), was ~10¹⁰ phage particles/ml of culture supernatant, which is typical for fd-tet derivatives. The phage yield obtained by titration is reproducibly underestimated by a factor 100. Assuming five EGF molecules displayed per phage, the number...
Figure 1. (A) Binding specificity of phage-EGF and negative control phage assayed by ELISA on coated cells. 2.2wt and A431 cells overexpress the EGFR (EGFR+ cells) and 2.2 cells do not express the EGFR (EGFR− cells). Phage titrations obtained by infection correspond to 10^9 p.f.u./ml. Titrations by ELISA on coated phages are represented with shaded bars. ELISA were repeated 5–10 times and error bars represent ±SEM. (B) Stimulation of DNA synthesis in NIH 3T3 fibroblasts. NIH 3T3 cells were serum-starved for 48 h and treated either with soluble EGF (a), phage-EGF (b) or phage control (c) and incubated for 18 h in the presence of 100 µM BrdU. BrdU incorporation was revealed by immunofluorescence staining with an anti-BrdU antibody. Nuclei were revealed by staining with 10 µM Hoechst dye.

of EGF displayed molecules therefore corresponds to 500× p.f.u. number, which is ~5 × 10^12 molecules/ml of phage preparation.

Phage-EGF and negative control phages displaying no protein or unrelated proteins were tested for binding to the EGFR by ELISA on cells (Fig. 1A). The assay was performed either on cells overexpressing the EGFR (2.2wt and A431 cells) or on cells not expressing the EGFR (2.2 cells). We ensured that all phages were used at the same concentration by bacteria infection titration and by direct ELISA detecting coated phages with an anti-phage antibody-HRP conjugated. For EGFR overexpressing cell lines, a strong ELISA signal was obtained with the phage-EGF and not with control phage. A low signal was obtained on cells that did not express the EGFR demonstrating the binding specificity to the EGFR.

We then tested the biological activity of phage-EGF by measuring its ability to induce DNA synthesis, which represents a late event (14–16 h) in the EGFR signal transduction pathway. Starved NIH 3T3 cells were treated with either soluble EGF (25 ng/ml), phage-EGF (10^10 p.f.u./ml) or control phage (10^10 p.f.u./ml). The total number of nuclei was visualised by Hoechst staining and DNA synthesis was measured by bromodeoxyuridine incorporation. As shown in Figure 1B, phage-EGF stimulation elicited DNA synthesis in 100% of cells, which is comparable to stimulation by soluble EGF. No DNA synthesis was detected in cells stimulated with phage control showing a specific stimulation by EGFR activation.

SIE-dependant luciferase expression as a reporter for EGFR activation

The biological activity of wild-type phage-EGF opens the possibility of screening activities of EGF mutants as phage displayed molecules, which can easily be purified through PEG precipitation.
from bacterial supernatants. However, the DNA synthesis assay described above is not suitable for the screening of a large panel of phage-EGF mutants. Therefore, we investigated earlier EGF responses to develop a workable assay for the EGFR transduction activity. To this aim, we devised a novel test, based on the induction of gene expression mediated by the c-fos gene enhancer SIE, which constitutes a marker for transduction through EGFR activation (30). The luciferase gene was chosen as a reporter.

We constructed the vector pZlucTKSIE, derived from pZlucTK vector (25), in which the luciferase gene reporter is expressed under the control of the TK promoter and three copies of the SIE element. COS-7 cells were transiently cotransfected with pRK5 EGF-R (27) and pZlucTKSIE constructs, serum starved for 24 h and stimulated for 5 h with soluble EGF. High levels of luciferase activity were obtained with soluble EGF (Fig. 2A). No luciferase expression was detected in COS-7 cells cotransfected with pRK5 EGF-R and pZlucTK, which lacks the SIE element, or transfected only with pZlucTKSIE. This indicated that both EGFR expression and the presence of SIE elements are necessary for luciferase expression. The implication of the SIE elements was demonstrated further by competition experiments (Fig. 2B). COS-7 cells were cotransfected with pRK5 EGF-R, pZlucTKSIE and increasing amounts of pKSSIE, a pBluescript KS(+) vector containing three copies of SIE. Luciferase activity was inhibited by 50% upon addition of a 20-fold molar excess of pKSSIE, and inhibited completely upon addition of a 100-fold molar excess, while no reduction was observed upon the same molar excess of pBluescript KS(+) vector containing no SIE copies.

We then assayed the ability of phage-EGF to activate luciferase expression (Fig. 2C). COS-7 cells cotransfected with pRK5 EGF-R and pZlucTKSIE constructs were serum starved for 24 h and stimulated for 5 h with soluble EGF (25 ng/ml), phage EGF (10^{10} p.f.u./ml) or control phage (10^{10} p.f.u./ml). Similar levels of luciferase activity were observed with soluble EGF or phage EGF, whereas stimulation with the control phage did not produce any luciferase expression.

The results obtained with transient transfectants show that luciferase expression depends on overexpression of EGFR and on the presence of the SIE element. The luciferase is therefore a suitable reporter gene for the monitoring of EGFR activation when put under the control of the TK promoter and the SIE enhancer cassette.

A 96-well plate luciferase assay

To avoid experimental variations due to transient transfections, we established 2.2 SIE cell line derived from 2.2 wt cells, stably expressing the pZlucTKSIE vector. We first determined the optimal EGF concentration for a 2 h stimulation through a dose response analysis in 2.2 SIE cells (Fig. 3A). Addition of 1 ng/ml EGF led to a 30-fold increase in luciferase activity above the background. The increase reached a maximum value of 200-fold for EGF concentrations between 100 and 200 ng/ml. We then studied luciferase expression in 2.2 SIE cells stimulated with 100 ng/ml of EGF (Fig. 3B). Luciferase expression was detectable 1 h after the stimulation and reached its maximum 4 h later. This agrees with previous reports on EGF signalling (30) and indicates that the induction of the luciferase reporter gene in 2.2 SIE is a good indicator for EGFR stimulation. A similar dose–response analysis was performed on 2.2 SIE cells using phage-EGF and control phage (Fig. 3C). Luciferase expression increased in proportion with increasing amounts of phage-EGF, whereas the control phage gave no detectable signal. Having set the experimental conditions to detect EGF stimulation, the assay was adapted for its use in a 96-well culture plate. Using this format, EGF and phage-EGF elicited a similar time course stimulation (Fig. 3D).

We also tested whether the luciferase assay could be used to detect EGF antagonists. A competition assay using either blocking (IgG 528) (31) or non-blocking (clone 29.1) (32)
Figure 4. Competition of EGF signalling detected with the luciferase reporter assay. (A) Resting 2.2 SIE cells were stimulated with soluble EGF (1 ng/ml) and an increasing amount (0.1–50 µg/ml) of either blocking (IgG 528) or non blocking (clone 29.1) anti-EGFR antibodies. (B) Resting 2.2 SIE cells were stimulated with phage-EGF and an increasing amount (0.1–50 µg/ml) of either blocking (IgG 528) or non blocking (clone 29.1) anti-EGFR antibodies. Luciferase is expressed as relative luciferase units (RLU). The experiment was repeated three times and error bars represent ±SEM.

anti-EGFR antibodies was performed (Fig. 4A and B). The blocking IgG 528 antibody was shown to prevent EGFR phosphorylation and subsequent signal transduction (31). The antibodies were mixed with EGF or phage-EGF before stimulation. Addition of the blocking antibody (1–50 µg/ml) completely abolished the luciferase signal elicited by either EGF or phage-EGF, whereas the non-blocking antibody (1–50 µg/ml) did not alter the signal. The use of our luciferase assay in 2.2 SIE cells therefore allows detection of agonists and antagonists.

Screening EGF mutants

The efficiency of the 96-well plate luciferase assay was then tested in the screening of a library of EGF mutants displayed on phage. Residue Asp46 was randomised to study the binding and transducing activity of the mutated EGF molecules. This residue was chosen because of its conservation throughout the EGF-like family of growth factors. Residue 46 links the second β-sheet of hEGF (residues 37–45) to the C-terminal helix (residues 47–51) (12). We constructed a D46 random library containing 10^4 clones, a number ensuring the presence of all possible mutants (33). Sequence analysis of 72 randomly picked clones revealed the presence of 18 different residues at this position (only glutamate and lysine were not found), indicating a good library complexity. Each mutant (titration and direct ELISA on coated phages were used to estimate the relative concentration of each phage), was tested in duplicate, either for binding to the EGFR by ELISA or for transduction activity using the luciferase assay (Fig. 5).

In all instances, the EGFR transducing activity of the mutated phages revealed by the luciferase assay paralleled the binding activity. Most mutants behaved similarly to the wild-type phage-EGF. Only leucine, isoleucine, histidine and arginine substitutions led to a reduction of both receptor binding and transduced signal. The cysteine substitution completely disrupted the binding to the EGFR, probably due to folding problems in the presence of the additional SH group. Our data show that replacement of the negative charge at position 46 by uncharged amino acids does not interfere with the binding, except for leucine and isoleucine. Effects observed for these two mutants might result from the length of their side chain, which may induce slight alterations in the C-terminal helix. Interestingly, replacement by positively charged amino acids (arginine and possibly histidine) have a clearly negative effect. Their presence might alter the conformation of both the following arginine at position 48 (through electrostatic repulsion) and as a consequence also the
tyrosine or arginine resulted in a decrease of relative EGFR affinity (14,16).

We tested a few mutants for their ability to induce DNA synthesis. Trp and Asn mutants, which are active when using our luciferase assay, induce DNA synthesis with an efficiency equivalent to the wild-type phage, whereas no BrdU incorporation was found when stimulating cells with Leu and Cys mutants (data not shown). The luciferase assay allows the identification of partial activities since the Leucine mutant is still able to induce 5% of luciferase expression, but it is not able to induce a later event such as DNA synthesis.

A previous report showed that replacement of Asp46 by alanine, tyrosine or arginine resulted in a decrease of relative EGFR affinity to 23, 14 and 4% respectively (34). Our results are in agreement as far as it concerns the arginine substitution, but differ for the alanine or tyrosine replacements. As according to both our assays, the mutated phage-EGF are still able to bind the EGFR as efficiently as the wild-type phage-EGF. The difference between our results obtained with phage displayed EGF and the previous results obtained with soluble mutants might be due to the phage multivalency. The three to five copies of EGF mutants displayed per phage are likely to increase the apparent binding efficiency through an avidity effect. To test this hypothesis, we randomised a more structural position, the arginine 41, directly involved in the EGFR binding site. The library was constructed and 36 sequences confirmed its complexity. Out of 192 clones assayed by ELISA, only 15 were able to bind to the EGFR showing that the binding is more severely affected. Sequence analysis of the bound phages revealed only wild-type phage encoding an arginine at the 41 position with three different possible codons (CGT, CGG or AGG). These results prove that phage display method is powerful in detecting crucial positions.

CONCLUSION

We showed that human EGF displayed on phage is able to transduce EGF growth signalling at least up to the DNA replication step and, therefore, that phage display can be used to study the biological activity of EGF mutants. Furthermore, a luciferase reporter assay was developed and proven to be a suitable tool for the analysis of EGFr signalling transduction. High signals were obtained with phage-EGF while no detectable signal was observed using irrelevant phages. Using previously characterised antibodies directed against the EGFR, the ability of the luciferase assay to detect EGFR antagonists has also been demonstrated. Analysis of a panel of EGF variants mutated at position 46 showed a good correlation between signal transduction activity as detected by the luciferase assay and cell binding as detected by phage ELISA. The randomisation of the 41 crucial position severely affected the binding as only wild-type phages were able to bind to the EGFR.

Besides the applicability of the luciferase reporter assay to screen for EGF agonists and antagonists, preliminary experiments indicate that the assay could also be used to study signal transduction by human TGFα (another EGFR ligand), by murine interferon-α and by other ligand/receptor complexes, whose signalling pathways include activation through the SIE elements.

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