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Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules

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The epidermal growth factor (EGF)-receptor is composed of an extracellular ligand-binding region connected by a single transmembrane region to the cytoplasmic kinase domain. In spite of its importance for understanding signal transduction, the ligand-binding domain of the EGF-receptor is not yet defined. We describe the identification of a major ligand-binding domain of the EGF-receptor by utilizing chimeras between the human EGF-receptor and the chicken EGF-receptor. This approach is based on the fact that murine EGF binds to the chicken EGF-receptor with 100-fold lower affinity as compared to the human EGF-receptor. Hence, the substitution of various domains of the chicken EGF-receptor by domains of the human EGF-receptor may restore the higher binding affinity towards EGF, characteristic of the human receptor. We show that chimeric chicken/human EGF-receptor, which contains domain III of the extracellular region of the human receptor, behaves like the human EGF-receptor with respect to EGF binding affinity and biological responsiveness. However, a chimeric chicken/human EGF-receptor containing domains I and II of the human receptor behaves like the chicken rather than the human EGF-receptor. Moreover, two different monoclonal antibodies which compete for the binding of EGF to EGF-receptor recognize specifically domain III of the human EGF-receptor. It is concluded that domain III which is flanked by the two cysteine-rich domains is a major ligand-binding domain of the EGF-receptor.

Key words: epidermal growth factor/receptor/chimeras ligand-binding domain

Introduction

The control of cell proliferation is regulated by specific interactions between growth factors and their cell surface receptors. The mitogenic response of epidermal growth factor (EGF) is transduced by its binding to the EGF-receptor; a membrane glycoprotein of mol. wt 170 000 daltons which possesses intrinsic protein tyrosine kinase activity (reviewed in Carpenter and Cohen, 1979; Schlessinger, 1986, 1988). The EGF-receptor is composed of an extracellular ligand-binding region connected by a single transmembrane region to the cytoplasmic kinase domain. Ligand binding to the extracellular domain stimulates the kinase activity which leads to receptor self-phosphorylation and to the phosphorylation of exogenous substrates (Ushiro and Cohen, 1980; Hunter and Cooper, 1985). Analyses of transfected cells expressing either wild-type or various EGF-receptor mutants indicated that the kinase activity of EGF-receptor is essential for signal transduction, normal receptor trafficking, stimulation of DNA synthesis and transformation (Livneh *et al.*, 1986, 1987; Honegger *et al.*, 1987a,b; Chen *et al.*, 1987; Moolenaar *et al.*, 1988).

In spite of its importance for understanding the mechanism of receptor activation, the ligand-binding region of neither the EGF-receptor nor any other growth factor receptor is defined. In our efforts to identify the ligand-binding region of EGF-receptor, we have affinity labeled the receptor with [^{125}I]EGF and identified a cyanogen bromide (CNBr) cleaved fragment containing the cross-linked EGF molecule (Lax *et al.*, 1988a). This CNBr-cleaved fragment is composed of 249 amino acids and it contains residues from both cysteine-rich domains and the entire region flanked by the two cysteine-rich domains.

Here we describe a functional characterization of the major ligand-binding region of the EGF-receptor by utilizing chimeric interspecies EGF-receptor molecules. This approach is based on the fact that mammalian EGF binds to the human EGF-receptor with 100-fold higher binding affinity than the chicken EGF-receptor when expressed in the same cellular background (Lax *et al.*, 1988b). The dose–response curves of various biological responses mediated by EGF were also shifted to higher concentrations in cells expressing the chicken EGF-receptor as compared to cells expressing the human receptor. One of the hallmarks of the extracellular domain of EGF-receptor is two cysteine-rich domains which are similar to cysteine-rich domains found in other receptor molecules such as insulin receptor, IGF_1 receptor and the putative receptor encoded by proto-oncogene *neu* (reviewed in Yarden and Ullrich, 1988). On the basis of internal sequence homology, the extracellular region of the EGF-receptor can be subdivided into four domains (Figure 1). Domain I is the amino-terminal domain, domain II and IV are the two cysteine-rich domains and domain III is flanked by the two cysteine-rich domains and possesses significant homology with domain I (Lax *et al.*, 1988b). We introduced unique restriction sites at the borders of these domains at identical positions in the cDNA of both human and chicken EGF-receptor. This allowed the replacement of various domains of the chicken EGF-receptor by the homologous domains from the human receptor. Here we show that a chimeric chicken/human (C/H) EGF-receptor containing domain III of the human EGF receptor behaves like the human EGF-receptor with respect to EGF binding affinity and biological responsiveness. Moerover, two different monoclonal antibodies which inhibit the binding of EGF bind specifically to domain III of the human EGF-receptor. It is concluded that domain III is a major ligand-binding region of the EGF-receptor.

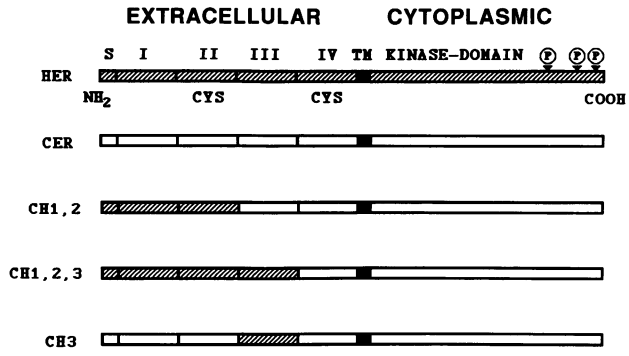

Fig. 1. Schematic representation of human, chicken and chimeric EGF-receptors. S denotes the signal sequence; CYS represent the two cysteine-rich domains (domains II and IV); TM the transmembrane region; P the known autophosphorylation sites Y1068, Y1148 and Y1173. HER—wild type human EGF-R, CER—wild type chicken EGF-R, CH1,2—chicken EGF-R containing domains I and II of human EGF-R. In this mutant residues 1–330 of CER were substituted by residues 1–324 of HER. CH1,2,3—chicken EGF-R containing domains I, II and III of human EGF-R. In this mutant residues 1–515 of CER were substituted by residues 1–508 of HER. CH3—chicken EGF-R containing domain III of human EGF-R. In this mutant residues 330–515 of CER were substituted by residues 324–508 of HER.

Results

Generation of cell lines expressing chicken, human or chicken/human chimeric EGF-receptors

Various cDNA constructs of EGF-receptor were generated (see Materials and methods) (Figure 1) and cloned into a mammalian expression vector (pLSV) which utilizes the SV40 early promoter to drive transcription (Livneh *et al.*, 1986). These constructs were generated with the following objectives: (i) unique restriction sites should be introduced at the borders of the putative structural domains and (ii) the restriction sites should be at homologous positions in chicken and human EGF receptors in order not to alter the amino acids present in the mutated regions. Figure 1 shows a schematic diagram of EGF-receptor constructs used in this study. Constructs containing the sequences of the entire human EGF-receptor or chicken EGF-receptor were termed HER and CER respectively. In chimeric EGF-receptor constructs, various regions from the extracellular region of the human (H) EGF-receptor were inserted to substitute for corresponding regions of the chicken (C) EGF-receptor (Figure 1). Hence, the chimeric chicken/human receptor termed CH1,2 contains domains I and II of the human receptor inserted to substitute for domains I and II of the chicken EGF-receptor, respectively (Figure 1). Similarly, chimera CH1,2,3 and chimera CH3 contain either domains I, II and III or domains III, respectively of the human EGF-receptor substituting the corresponding domains of the chicken EGF-receptor.

The cDNA constructs encoding the various EGF-receptors were co-transfected with neomycin resistance gene (pSVNeo) into NIH-3T3 cells lacking endogenous EGF-receptors (Honegger *et al.*, 1987a,b; Lax *et al.*, 1988b). After selection with Geneticin (G418) the cloned cell lines were screened for the expression of EGF-receptor utilizing immunoprecipitation experiments of phosphorylated EGF-receptor using anti-EGF-receptor antibodies RK2 (Kris *et al.*, 1985) which recognize both the chicken and the human EGF-receptors (Lax *et al.*, 1988b).

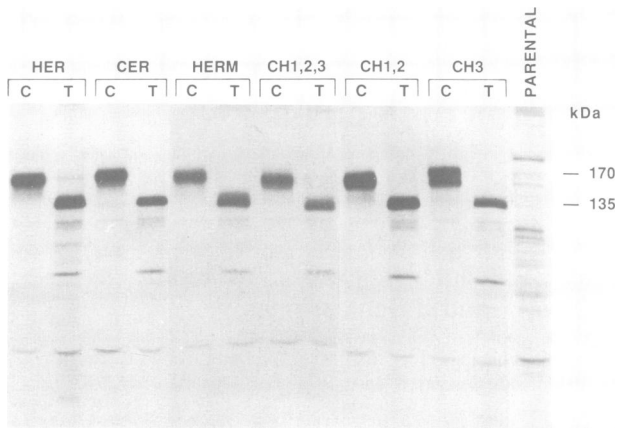

Fig. 2. Identification of human, chicken and the chimeric EGF-receptors by immunoprecipitation of [^{35}S]methionine-labeled cells. Labeled cells were treated in the absence (C) or presence (T) of tunicamycin for 12 h at 37°C then lysed and immunoprecipitated with anti-EGF-receptor antibodies (RK-2). The samples were analysed by SDS–PAGE using a 7.5% gel and autoradiography. The autoradiograph shows, in addition to HER and CER, a human EGF-receptor containing unique restriction sites denoted HERM which was used to engineer the various chicken/human chimeric receptors CH1,2; CH1,2,3 and CH3. Labeling of cells with [^{35}S]methionine, immunoprecipitation of labeled EGF-receptor with RK-2 anti-EGF-receptor antibodies (Kris *et al.*, 1985) and separation by SDS–PAGE were done according to published procedures (Honegger *et al.*, 1987a; Lax *et al.*, 1988a,b).

Table I. Binding parameters of EGF-receptor mutants for EGF and TGF-α

Cell line (receptors/cell)	*Kd* for EGF (M)	*Kd* for TGF-α (M)
HER (6.0×10^5)	0.8×10^{-9}	1.8×10^{-9}
CER (1.5×10^5)	2.6×10^{-7}	0.9×10^{-9}
CH1,2 (8×10^5)	2.6×10^{-7}	2.3×10^{-9}
CH1,2,3 (1.8×10^5)	0.5×10^{-9}	1.3×10^{-9}
CH3 (0.9×10^5)	1.6×10^{-9}	1.1×10^{-9}

TGF-α binding to human or chicken EGF-receptors displays a single binding constant (Lax *et al.*, 1988b). Dissociation constants (*Kd*) for EGF and TGF-α were determined from displacement curves of ^{125}I-labeled TGF-α either by EGF or TGF-α as described in Figure 3. Each experiment was repeated three times with essentially the same results.

Biosynthetically, [^{35}S]methionine-labeled cells expressing either chicken, human or chimeric C/H EGF-receptors treated in the absence or presence of tunicamycin, were subjected to immunoprecipitation experiments and then analysed by SDS–PAGE and autoradiography (Figure 2). Both human, chicken and the various chimeric C/H receptors were all expressed as a glycoprotein of mol. wt 170 000 daltons. In the presence of tunicamycin, which inhibits receptor glycosylation, all the receptor molecules had a mol. wt of 135 000 daltons indicating that the protein backbone of the chicken, human or chimeric receptors were similar. All the experiments described in this study were repeated with at least two different cell lines, obtained from independent transfection experiments.

Binding experiments with [^{125}I]EGF or with [^{125}I]TGF-α

The surface display of HER, CER or the various chimeric receptors was first revealed by binding experiments with

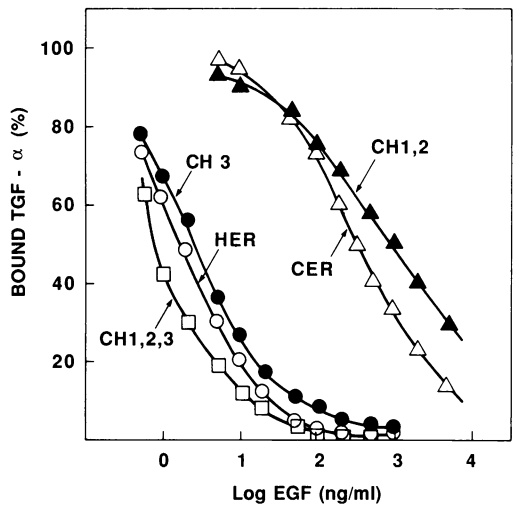

Fig. 3. Inhibition of binding of ^{125}I-labeled TGF-α by native EGF to cells expressing human, chicken and chimeric EGF-receptors. Various cell lines expressing human, chicken or chimeric receptors were incubated with a solution containing increasing concentrations of native EGF and 20 ng/ml of ^{125}I-labeled TGF-α for 1 h at room temperature (Lax *et al.*, 1988b). After several washes with DMEM containing 0.1% BSA, the cells were lysed, and the cell associated radioactivity (bound [^{125}I]TGF-α) was determined for every cell line. Similar results were obtained when the experiments were performed at 4°C. HER (○); CER (Δ), CH1,2 (▲); CH1,2,3 (□) and CH3 (●).

^{125}I labeled TGF-α. We have previously shown that human [^{125}I]TGF-α binds equally well to the human and chicken EGF-receptors whereas murine EGF binds with 100-fold reduced affinity to the chicken receptor as compared to the human receptor (Lax *et al.*, 1988b). Analysis of binding experiments with ^{125}I-labeled TGF-α according to the method of Scatchard revealed a single dissociation constant for the human, chicken and chimeric C/H EGF-receptors with similar *K*d values in the range of $1-2.3 \times 10^{-9}$ M (Table I). This result indicates that all the chimeras retain the binding activity of the parent molecules towards TGF-α and the exchange of domains did not impair the affinity of the chimeric receptors towards TGF-α. The binding affinity of murine [^{125}I]EGF towards chicken EGF-receptor is too low to be determined by conventional binding experiments and Scatchard analysis. Therefore, the binding affinity of EGF to the chicken EGF receptor was determined by a displacement analysis in which cell-bound [^{125}I]TGF-α was displaced by increasing concentrations of native EGF (Lax *et al.*, 1988b). Using this approach, we show a remarkable increase in EGF binding affinity by chimera CH1,2,3 and CH3, but not in chimera CH1,2 (Figure 3). The apparent *K*d of EGF to the human receptor is 0.8×10^{-9} M while the *K*d of EGF to the chicken EGF-receptor is 2.6×10^{-7} M. Chimeric receptor CH1,2 had a *K*d similar to the *K*d of chick EGF-receptor (2.6×10^{-7} M) while chimeras CH1,2,3 and CH3 had a *K*d similar to the *K*d of the human EGF-receptor, 0.5×10^{-9} M and 1.6×10^{-9} M respectively (Table I). These results were confirmed by direct binding experiments with [^{125}I]EGF to the same cell lines demonstrating that HER, CH1,2,3 and CH3 have similar EGF binding characteristics (Figure 4). Namely, like wild-type human EGF-receptors, or native EGF-receptor chimeras CH3 and CH1,2,3 were displayed on the cell surface with typical high [*K*d = $0.5-1 \times 10^{-10}$ M, 2−5%] and low [*K*d = $10-25 \times 10^{-9}$ M, 95−98%] affinity binding sites

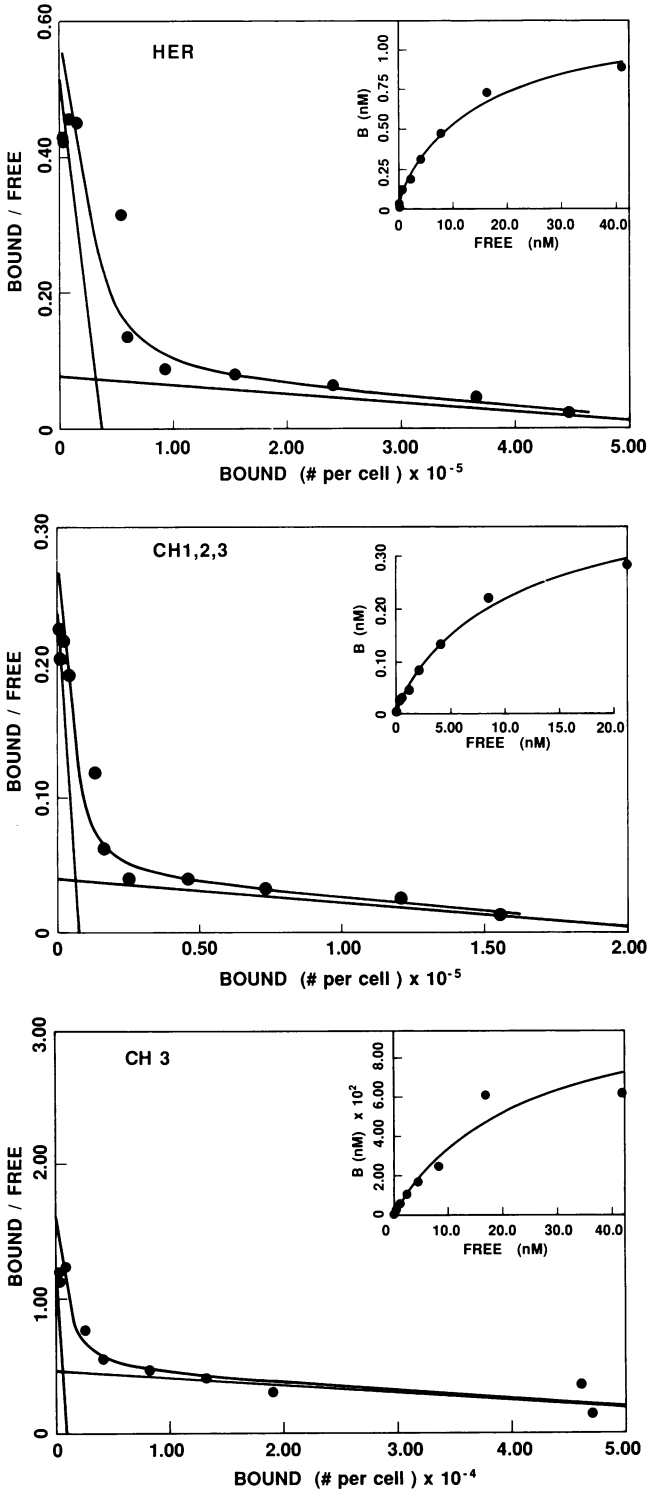

Fig. 4. Scatchard analysis of [^{125}I]EGF binding to cells expressing human and chimeric receptors. [^{125}I]EGF binding was determined for concentrations of [^{125}I] ranging from 0.06 to 600 ng/ml after 60 min incubation at room temperature. Non-specific binding was determined by parallel binding experiments to parental NIH-3T3 cells which lack endogenous EGF-receptors. The binding data are analysed according to the method of Scatchard. Scatchard plots and binding curves (in inserts) are shown for cell lines expressing HER, CH1,2,3 and CH3 receptors. The binding affinity of EGF to cells expressing CER and CH1,2 were too low to be determined by this approach.

for [^{125}I]EGF (reviewed in Schlessinger 1986, 1988). It was, therefore, concluded that chimeric receptor CH1,2,3, or CH3, but not CH1,2 have [^{125}I]EGF binding properties

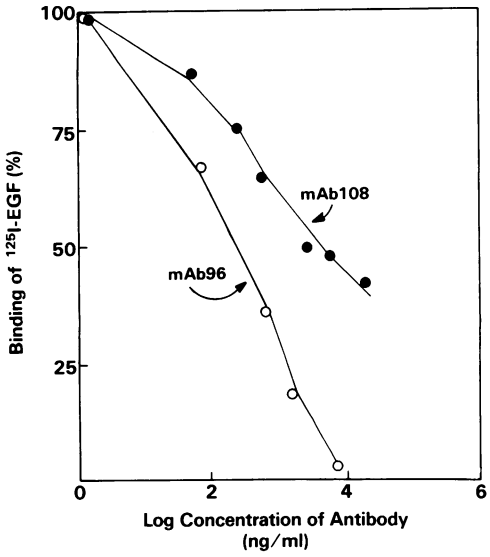

Fig. 5. Inhibition of binding of [^{125}I]EGF by monoclonal antibodies. NIH-3T3 cells expressing wild-type human EGF receptor were incubated with 20 ng/ml of [^{125}I]EGF for 1 h at room temperature with increasing concentrations of either monoclonal antibody mAb108 (●) or mAb96 (○). After three washes with PBS the cells were solubilized and their radioactivity determined. The *kd* values of mAb108 and mAb96 are 2.5 nM and 0.4 nM respectively. This experiment was repeated three times with essentially the same results.

of the wild-type human EGF-receptor. These results indicate that a major binding site for EGF resides in domain III of the EGF-receptor.

Analysis with monoclonal anti EGF-receptor antibodies

We have generated several different monoclonal antibodies which bind to the extracellular domain of the human EGF-receptor but not to the chicken or the murine EGF-receptors (Bellot *et al.*, in preparation). Two different monoclonal antibodies which do not influence each other's binding are able to compete with [^{125}I]EGF for binding to the EGF-receptor. While mAb96 is a very potent inhibitor of EGF binding, mAb108 has only a partial effect on the binding of [^{125}I] EGF to the human EGF-receptor (Figure 5). To analyse the region in the EGF-receptor recognized by these antibodies, we have compared their ability to immunoprecipitate HER, CER or the various C/H chimeric EGF-receptors expressed in the transfected cells. Figure 6 shows that both monoclonal antibodies mAb108 and mAb96 are able to specifically immunoprecipitate phosphorylated HER, CH1,2,3 and CH3 but not CER and CH1,2. Hence, these two monoclonal antibodies are directed against epitopes located in domain III of the human EGF-receptor. Since both antibodies compete for the binding of EGF to the receptor, these results provide further support for the role of domain III in determining ligand binding specificity of EGF.

Biological responses mediated by chimeric chicken/human EGF-receptors

We have tested whether the enhanced binding affinities of chimeras CH3 and CH1,2,3 also increased the sensitivity of these two receptor mutants to EGF by shifting to lower concentrations the dose−response curves for the stimulation of various biological receptors by EGF.

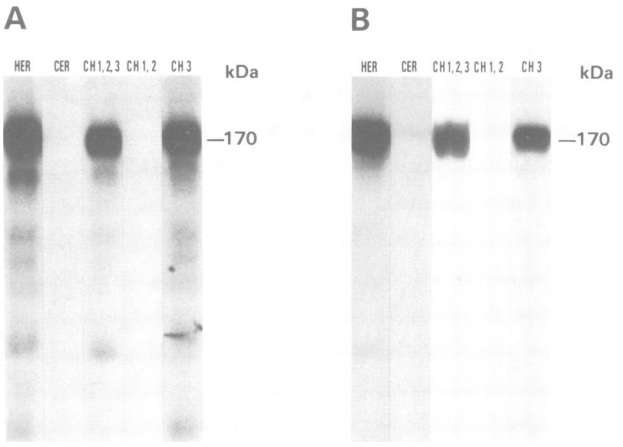

Fig. 6. Immunoprecipitation of human, chicken or chimeric EGF-receptors by monoclonal antibodies. Transfected NIH-3T3 cells expressing HER, CER and various C/H chimeric receptors were lysed and subjected to immunoprecipitation experiments with either mAb108 or mAb96. The samples were analysed by SDS−PAGE followed by autoradiography.

Tyrosine phosphorylation in living cells. Transfected cells expressing CER, HER or various C/H chimeric receptors were incubated with EGF for 15 min at 37°C. After solubilization, immunoprecipitation with anti EGF-receptor antibodies RK-2 (Kris *et al.*, 1985) and electrophoretic separation by SDS−PAGE the samples were transferred to nitrocellulose filter and probed with anti-phosphotyrosine antibodies and ^{125}I-labeled protein-A (Naldini *et al.*, 1986). Figure 7 shows that autophosphorylation of CER and CH1,2 was observed only upon addition of 500 ng/ml of EGF while autophosphorylation of HER, CH1,2,3 and CH3 was already observed at 5 ng/ml of EGF. Chimeric receptor CH1,2,3 is more potent than CH3 in EGF-stimulated autophosphorylation. This is consistent with the 2- to 3-fold increased binding affinity of CH1,2,3 as compared to CH3, as determined by [^{125}I]TGF-α displacement analysis (Figure 3 and Table I).

Receptor down-regulation. The transfected cells expressing CER, HER or the various C/H chimeric receptors were incubated with different concentrations of EGF for 2 h at 37°C. After careful washings and incubation for 45 min at 37°C to assure release of surface bound EGF, the amount of EGF-receptor on the cell surface was determined by quantitative binding experiments with [^{125}I]TGF-α which binds equally well to HER, CER and the three chimeric C/H receptors. Table II shows that 500 ng/ml of EGF induced 65−68% down-regulation of CER and CH1,2 after 2 h incubation, while in cell lines containing HER, CH3 and CH1,2,3 five ng/ml of EGF induced 45−60% down-regulation of receptors after 2 h of incubation at 37°C. Clearly, the higher binding affinity of CH1,2,3 and CH3 renders these receptor mutants more responsive to lower concentrations of EGF.

DNA synthesis. We have compared the capacity of EGF to stimulate DNA synthesis in NIH-3T3 cells expressing HER, CER and the various C/H chimeric receptors. Table III shows that the half maximal stimulation of DNA synthesis was accomplished at 1−3 ng/ml of EGF for cells expressing

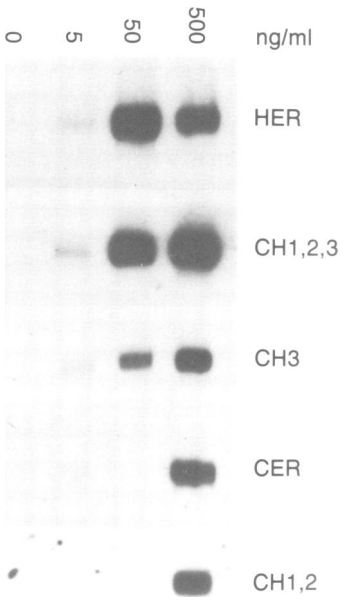

Fig. 7. Autophosphorylation of human, chicken and chimeric receptors in living cells. Cells expressing either wild-type or mutant receptors were exposed to 5, 50 or 500 ng/ml of EGF respectively for 15 min at 37°C followed by cell solubilization, electrophoretic separation on SDS–gel, blotting and analysis with anti-phosphotyrosine antibodies according to published procedures (Naldini *et al.*, 1986).

Table II. Down-regulation of human, chicken or chimeric EGF-receptors

EGF-receptor mutants	Concentration of EGF used to induce down-regulation (%)		
	5 ng/ml	10 ng/ml	500 ng/ml
CER	5%	4%	65%
CH1,2	6%	5%	68%
CH1,2,3	60%	60%	80%
CH3	45%	52%	80%
HER	52%	55%	85%

Various cell lines were incubated with different concentrations of EGF for 2 h at 37°C. The cells were carefully washed with DMEM containing 0.1% BSA and further incubated for 45 min at 37°C. To assure release of surface-bound EGF, the amount of surface receptors were determined by binding experiments with ^{125}I-labeled TGF-α (20 ng/ml for 1 h at room temperature) which binds equally well to the chicken, human and chimeric EGF-receptors. This experiment was repeated four times with essentially the same results.

HER, CH1,2,3 and CH3 while 100–300 ng/ml of EGF was required to achieve a similar level of DNA synthesis in NIH-3T3 cells expressing CER or CH1,2.

On the basis of this analysis we have concluded that the dose–response curves for the various responses of EGF were all shifted to lower concentrations of the growth factor for NIH-3T3 cells expressing CH1,2,3 or CH3 chimera similar to the dose used to achieve a biological response in cells expressing the human EGF-receptor.

Discussion

We have constructed a chicken EGF-receptor mutant which has EGF-binding and biological properties similar to those

Table III. Stimulation of DNA synthesis

EGF-receptor mutants	Half-maximal stimulation by EGF (ng/ml)	Stimulation ratio[a]
HER	1–3	5–7
CER	100–250	6–9
CH1,2	100–300	5–7
CH1,2,3	1–2	6–7
CH3	1–3	5–7

[a]Stimulation ratio is the maximal thymidine c.p.m. incorporated in the presence of growth factor divided by the incorporated c.p.m. in the absence of growth factor. These are the results of three experiments performed in duplicate, demonstrating the variability in DNA synthesis experiments of cultured cells. Similar variability is observed in cells expressing native EGF-receptors.

of the human EGF-receptor expressed in the same cellular background. This was achieved by exchanging domain III (Figure 1) of the human receptor with the corresponding region of the chicken EGF-receptor. Chimeras which contained domains I and II of the human receptor did not show enhanced affinity for EGF but retained the high affinity of the chicken receptor towards TGF-α. This result demonstrates that, despite the lack of relevant information about the three-dimensional structure of the EGF-receptor, we have succeeded in generating functional chimeric receptors which retained the binding properties of the parental molecules towards TGF-α. The exchange of domains did not disturb the overall folding of the receptor chimera, further supporting the hypothesis of domain structure of the EGF-receptor (Lax *et al.*, 1988b). The C/H chimera are also useful for mapping antigenic epitopes which react with monoclonal anti EGF-receptor antibodies. Chimeric EGF receptor CH3 is specifically recognized by monoclonal antibodies which compete with [^{125}I]EGF for binding to the human EGF receptor. The monoclonal antibodies do not recognize the chicken EGF-receptor and chimera CH1,2. On the basis of these results, we propose that domain III plays a crucial role in defining ligand binding specificity and the display of high affinity EGF-receptors. Although it is possible that domain III has an indirect influence on the ligand binding affinity of EGF, we favor the explanation that domain III of EGF-receptor contributes directly most of the interactions which define EGF binding specificity.

On the basis of internal sequence homology, we have previously reasoned that the extracellular region of the EGF-receptor is composed of four subdomains which we have termed domains I–IV (Lax *et al.*, 1988a,b). We have isolated a large CNBr-cleaved affinity labeled polypeptide containing 249 amino acids which encompasses the entire domain III (150 amino acids) and an additional 100 amino acids from the two flanking cysteine-rich domains (Lax *et al.*, 1988a). This analysis indicates that in the occupied EGF-receptor certain residues of EGF are in close proximity to amino acid residues in the CNBr-cleaved fragment of EGF-receptor. Yet, we cannot rule out possible contributions from other domains of EGF-receptor for EGF binding.

In this study, we analysed the function of individual domains by exchanging DNA pieces which correspond to putative domains without flanking residues from cysteine-rich domains. Three independent lines of evidence point to

domain III as the major contributor to EGF binding site: (i) human domain III increases 100-fold the binding affinity of chimeric C/H EGF-receptor towards EGF, (ii) monoclonal antibodies which inhibit EGF binding recognize specifically human domain III; and (iii) domain III is part of a larger affinity-labeled CNBr-cleaved fragment (Lax *et al.*, 1988a). Once the affinity of the chicken EGF-receptor towards EGF is improved by exchange of domain III with the human EGF-receptor, the dose–response curves for various biological responses are shifted to a lower concentration of EGF. Comparison of domains III of human and chicken EGF-receptors (Lax *et al.*, 1988b) revealed 75% sequence identity. Hence, additional analysis is required to identify which of the different 35 amino acids of domain III of HER play a role in the generation of high affinity EGF binding. Yet, chimera CH1,2,3 has ~3-fold higher binding affinity and it also mediates biological effects of EGF as, or even more effectively than, wild-type human EGF-receptor expressed in the same cellular background. It is likely that other domains, particularly domain I contribute additional direct interactions required to bring about full affinity of EGF-receptor towards EGF. Alternatively, domains I and II may influence the conformation of domain III indirectly rendering it a more potent receptor towards EGF. Further analysis using additional chimeric receptors is required in order to resolve between these two possibilities.

The experiments using monoclonal antibodies warrant further discussion. First, both monoclonal antibodies inhibit EGF in an equimolar ratio, suggesting that the antibodies either compete for the same site or bind to a site which is in close proximity to the EGF-binding site. Second, the two antibodies described in this study, mAb108 and mAb96, and two additional monoclonal antibodies which do not affect EGF binding (Bellot *et al.*, in preparation), all recognize specifically domain III of the human EGF-receptor. Hence, domain III of the human EGF receptor contains antigenic determinants which are probably more exposed than other domains, thus rendering them immunodominant. This notion is also consistent with the view that domain III plays an important role in defining ligand-binding specificity for EGF.

The EGF-receptor is a member of the PTK family of growth factor receptors. The extracellular domains of the product of the proto-oncogene *neu* (HER2), the insulin-receptor and IGF_1 receptor all contain cysteine-rich domains and domains reminiscent of domain III of the EGF-receptor (reviewed in Yarden and Ullrich, 1988). On the basis of the current analysis, we propose that regions equivalent to domain III in other growth factor receptors may play a role in defining ligand-binding specificities in the family of receptors. Hence, similar experiments performed for other growth factor receptors may help in identifying the regions which define their ligand-binding specificities.

Materials and methods

Preparation of constructs

To generate C/H chimeras, we introduced two unique restriction sites, *Sna*BI at the end of domain II and *Mlu*I at the beginning of domain IV (Figure 1), at homologous positions in both human and chicken EGF-receptor cDNA. The *Sna*BI site was introduced at nucleotide 1282 and 1156 of CER and HER respectively (Ullrich *et al.*, 1984; Lax *et al.*, 1988b), and the *Mlu*I site was introduced at position 1832 and 1706 of CER and HER respectively. For the *Sna*BI site the oligonucleotides GAAAATGGTGTACGTAAGTGT and GAAGACGGCGTACGTAAGTGT were used to mutate CER and HER respectively (the mutated nucleotides are underlined). These mutations did not change the codons of either CER or HER. For the *Mlu*I site the oligonucleotides GTGTGTGACGCGTTGTGCTCG and GTCTGCCACGCGTTGTGCTCC were used to mutate CER and HER respectively. The mutation in CER changed Pro-485 to Ala which is present at this position in HER. Site-directed mutagenesis was performed on single-stranded M13um20 (International Biotechnologies, Inc., New Haven, CT) clones containing the entire CER or HER insert as an *Xho*I fragment. The two oligonucleotides designed to generate *Sna*BI and *Mlu*I sites were phosphorylated and used simultaneously as primers for annealing to the respective M13 DNA followed by extension with the Klenow enzyme and ligation, according to the procedure described by Gillam and Smith (1979a,b). After transfection to JM103 the mutant phages were detected by hybridization of the M13 plaques with labeled oligonucleotides and confirmed by DNA sequencing. The mutated receptor DNA insert was isolated from the replicative forms of the phages as an *Xho*I fragment and ligated into pLSV as described (Livneh *et al.*, 1986; Lax *et al.*, 1988b). These constructs were denoted CERM and HERM for mutated CER and mutated HER, respectively. The chicken/human chimera CH3 was generated by ligating the *Sna*BI–*Mlu*I fragment (0.55 kbp) from HERM into CERM from which the *Sna*BI–*Mlu*I fragment was removed by agarose gel electrophoresis. Chimera CH1,2 was prepared by ligating the *Kpn*I–*Sna*BI (1.3 kbp, *Kpn*I is a unique site in the pLSV vector 366 bp upstream from the 5′ end of the receptor cDNA) from HERM into CERM from which the *Kpn*I–*Sna*BI fragment was removed by agarose gel electrophoresis. Chimera CH1,2,3 was similarly prepared by exchanging the *Kpn*I–*Mlu*I of CERM (1.8 kbp) with that of HERM. The resultant plasmids were prepared by the CsCl method and used to transfect NIH-3T3 cells (Wigler *et al.*, 1979).

[^{35}S]Methionine labeling

Subconfluent cells in 10 cm dishes were washed with methionine- and cysteine-free Dulbecco's modified Eagle's medium (DMEM) and grown for 12 h in methionine- and cysteine-free DMEM/10% fetal calf serum (FCS) containing 50 uCi/ml of [^{35}S]methionine and [^{35}S]cysteine. The cells were washed three times with phosphate-buffered saline (PBS), then scraped into 0.5 ml of lysis buffer [20 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1 mM EGTA; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 mM phenylmethylsulphonyl fluoride (PMSF)], incubated for 5 min on ice, and then the lysate was spun for 30 min in an Eppendorf centrifuge in the cold. Three micrograms of protein A–Sepharose per sample was suspended in 20 mM Hepes (pH 7.5), washed with 20 mM Hepes, and incubated for 30 min at room temperature with anti-EGF-receptor antibodies (RK-2). The protein A–Sepharose/antibody complex was washed three times with HNTG (20 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 0.1% Triton X-100) and incubated with the cell lysate for 90 min at 4°C. The immunoprecipitate was then washed twice with 50 mM Hepes (pH 8.0), 0.2% Triton X-100, 500 nM NaCl, and 5 mM EGTA; once with HNTG buffer, and twice with 50 mM Hepes (pH 8.0), 0.1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EGTA; and twice with 10 mM Tris–HCl (pH 8.0), 0.1% Triton X-100. Then, 3 vols of sample buffer were added to the washed immunoprecipitate, boiled for 4 min, and electrophoretically separated on a 7.5% SDS–polyacrylamide gel.

Binding experiments

For all [^{125}I]EGF or [^{125}I]TGF-α binding assays, cells were plated at a density of 100 000 cells per well in 24-well dishes coated with 10 μg per well of human plasma fibronectin (Meloy Laboratory), and were allowed to grow for 48 h to confluency in DMEM containing 10% FCS. Mouse EGF (Toyobo, Tokyo) or human TGF-α (Genentech, South San Francisco) were iodinated by using the chloramine-T method to a specific activity of 100 000–200 000 c.p.m./ng. Confluent cells were washed with DMEM containing 1 mg/ml bovine serum albumin (BSA), and were then incubated with either [^{125}I]EGF or [^{125}I]TGF-α in the same buffer. Non-specific binding was determined by parallel binding experiments to parental cells which are devoid of EGF-receptors. After incubation for 60 min at room temperature the cells were placed on ice and washed three times with ice-cold PBS containing 1 mg/ml of BSA. The cells were lysed in 0.5 ml of 0.5 M NaOH for 30 min at 37°C, and the radioactivity was measured in a gamma counter to determine the amount of ligand bound to the cell surface.

Tyrosine phosphorylation in living cells

Cells were grown to confluence in 10 cm dishes, starved overnight in DMEM containing 0.5% calf serum and stimulated for 15 min with various concentrations of EGF. The cells were lysed in 0.5 ml of 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin and leupeptin, 100 mM sodium fluoride, 30 mM *p*-nitrophenyl phosphate, 10 mM sodium pyrophosphate and 0.2 mM sodium orthovanadate and centrifuged for 15 min at 10 000 *g*.

The EGF-receptor was immunoprecipitated from the supernatant using RK2 anti-EGF-receptor antibodies, analysed on two 7% SDS–polyacrylamide gels and transferred to nitrocelluose for immunoblotting with anti-phosphotyrosine antibodies (gift from P.Comoglio). [^{125}I]protein A was used to detect the antibodies by autoradiography.

[^{3}H]Thymidine incorporation

Cells were seeded at a density of 100 000 cells/well in fibronectin-coated 24-well Costar dishes and grown for 2 days in 10% calf serum, then starved for 2 days in 0.5% calf serum. EGF or fetal calf serum was added and the cells were incubated for 18 h. [^{3}H]Thymidine was added and after 4 h, the cells were washed three times with PBS, incubated with ice-cold 5% trichloroacetic acid (TCA) for 30 min on ice and washed three times with PBS. The TCA precipitate was solubilized in 0.5 N NaOH for 30 min at 37°C and counted in a scintillation counter.

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