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Noncontiguous regions in the extracellular domain of EGF receptor define ligand-binding specificity

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Murine epidermal growth factor (EGF) binds with ~250-fold higher binding affinity to the human EGF receptor (EGFR) than to the chicken EGFR. This difference in binding affinity enabled the identification of a major ligand-binding domain for EGF by studying the binding properties of various chicken/human EGFR chimera expressed in transfected cells lacking endogenous EGFR. It was shown that domain III of EGFR is a major ligand-binding region. Here, we analyze the binding properties of novel chicken/human chimera to further delineate the contact sequences in domain III and to assess the role of other regions of EGFR for their contribution to the display of high-affinity EGF binding. The chimeric receptors include chicken EGFR containing domain I of the human EGFR, chicken receptor containing domain I and III of the human EGFR, and two chimeric chicken EGFR containing either the amino terminal or the carboxy terminal halves of domain III of human EGFR, respectively. In addition, the binding of various human-specific anti-EGFR monoclonal antibodies that interfere with EGF binding is also compared. It is concluded that noncontiguous regions of the EGFR contribute additively to the binding of EGF. Each of the two halves of domain III has a similar contribution to the binding energy, and the sum of both is close to that of the entire domain III. This suggests that the folding of domain III juxtaposes sequences that together constitute the ligand-binding site. Domain I also provides a contribution to the binding energy, and the added contributions of both domain I and III to the binding energy generate the high-affinity binding site typical of human EGFR.

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Introduction

Epidermal growth factor (EGF) mediates its mitogenic response by binding to and activating an integral membrane protein, the EGF receptor (EGFR) (reviewed in Ullrich and Schlessinger, 1990). The EGFR is composed of three major structural domains; an extracellular ligand binding domain; a single hydrophobic transmembrane region; and a cytoplasmic domain containing, among other regions, a protein tyrosine kinase domain (Ullrich and Schlessinger, 1990). The extracellular ligand-binding domain can be further subdivided to four subdomains. According to this assignment, domain I is the amino terminal domain, domains II and IV are the two cysteine rich domains, and domain III is located between these latter cysteine rich domains (Lax *et al.,* 1988–1990). Sequence alignment of domains I with III and II with IV reveals significant sequence identity, suggesting that these domains may have evolved from an ancestral gene-by-gene duplication. In our efforts to identify the ligand-binding domain of EGFR and to elucidate the nature of the interactions that define ligand-binding specificity, we have used both chemical and genetic approaches. First, EGFR was affinity labeled with ^{125}I-EGF; and the cyanogen bromide (CNBr)-cleaved fragment, which contained the labeled ligand, has been identified to be domain III of EGFR (Lax *et al.,* 1988). Second, various mutant EGFRs were analyzed in transfected cells for EGF binding and biological responsiveness. It was demonstrated that deletion mutants devoid of domain I bind EGF with ~10-fold lower binding affinity than does solubilized wild-type EGFR (Lax *et al.,* 1990). Finally, we had generated chimeric chicken/human (C/H) EGFRs in which domains of chicken (C) EGFR were replaced by corresponding domains of the human (H) EGFR, resulting in EGFR chimera with high binding affinity toward EGF (Lax *et al.,* 1989). Using this approach, we have shown that domain III of EGFR is a major ligand-binding domain. To de-

lineate more precisely the binding determining sequence in domain III and to identify additional ligand-binding regions, we generated new C/H chimera and analyzed their binding properties toward EGF. Here we show that several noncontiguous regions in domains I and III play crucial roles in the interactions that give rise to high-affinity binding of EGF.

Results

The cDNA constructs encoding the new C/H chimera were cloned into the mammalian expression vector PLSV, which uses the SV40 early promoter to drive transcription. A schematic diagram of EGFR constructs used in this study is shown in Figure 1. Constructs containing either human or chicken EGFR sequences exclusively were termed HER and CER, respectively. Chimera CH1,2,3, CH1,2, and CH3 were previously described (Lax *et al.,* 1989), and cell lines expressing these C/H chimera were used for comparison with the new chimeras. In the C/H chimeric receptor designated CH1, domain I (residues 1–163) of human EGFR was substituted for domain I (residues 1–164) of chicken EGFR (Figure 1). Similarly, chimera CH1,3 contains both domains I (residues 1–163) and III (324–508) of human EGFR, substituting the corresponding domains of the chicken EGFR (residues 1–164 and 330–515, respectively). In chimeric receptor CH3A, the 5′ half of domain III of human EGFR (residues 324–423) replaced the corresponding domain of the chicken EGFR (residues 330–430). Similarly, chimera CH3B contains the 3′ half of domain III of human EGFR (residues 424–508), replacing the corresponding region of the chicken EGFR (residues 431–515). The expression vectors containing the cDNA constructs encoding the chimeric receptors CH1, CH1,3, CH3A, and CH3B were cotransfected along with a vector containing the neomycin resistance gene (pSVNeo) into NIH 3T3 cells lacking endogenous EGFR (Honegger *et al.,* 1987a,b; Lax *et al.,* 1988b), followed by selection of transfected cells with Geneticin (G-418). Resistant clones were screened for the expression of EGFR by the use of a standard immunoprecipitation/autophosphorylation assay (Lax *et al.,* 1988b) employing anti-EGFR RK2 antibodies (Kris *et al.,* 1985) that recognize chicken and human EGFR equally well (Lax *et al.,* 1988b).

Expression of the new C/H chimera was analyzed by immunoprecipitation experiments with RK2 anti-EGFR antibodies after ^{35}S-methionine and cysteine labeling. Treatment with tunicamycin, which prevents glycosylation, was used to assess the molecular mass of the protein core of chimeric receptors. Figure 2 shows that all of the new chimeric receptors—the previously described chimeric receptors as well as wild-type chicken (CER) and human receptors (HER)—migrated on sodium dodecyl sulfate (SDS) gels with apparent molecular mass of ~170 000. Because of tunicamycin treatment the various receptors migrated with apparent molecular mass of ~135 000. We have selected several cell lines of each chimeric receptor from several transfections and chosen to explore the properties of cell lines that were optimally matched for the level of receptor expression.

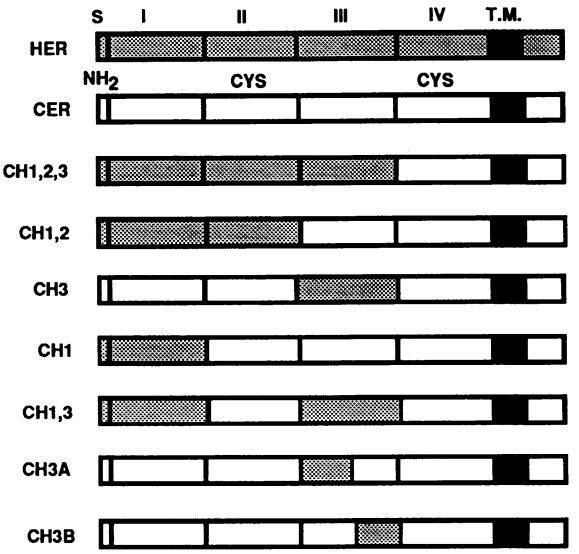

***Figure 1.* Schematic representation of human, chicken, and chimeric EGFRs.** S denotes the signal sequence; CYS, the two cysteine-rich domains (domains II and IV); TM, the transmembrane region; HER, wild-type human EGFR; CER, wild-type chicken EGFR. The chimeric receptors CH1,2,3, CH1,2, and CH3 were described (Lax *et al.,* 1989). CH1, chicken EGFR containing domain I of the human EGFR; CH1,3, chicken EGFR containing domains I and III of the human EGFR; CH3A, chicken EGFR containing the half 5′ region of human domain III; CH3B, chicken EGFR containing the half 3′ region of human domain III. Although the carboxy-termini of the receptor in the figure are truncated, the actual receptors extend to their natural carboxy-termini.

We had previously shown that transforming growth factor-α (TGF-α) binds with similar high binding affinity to both human and chicken EGFRs, whereas EGF binds with ~250-fold reduced binding affinity to the chicken EGFR compared with the human EGFR (Lax *et al.,* 1988b). We have, therefore, first determined the binding affinity of ^{125}I-TGF-α to the new chimeric receptors and analyzed the binding data according to the method of Scatchard (1949). Ta-

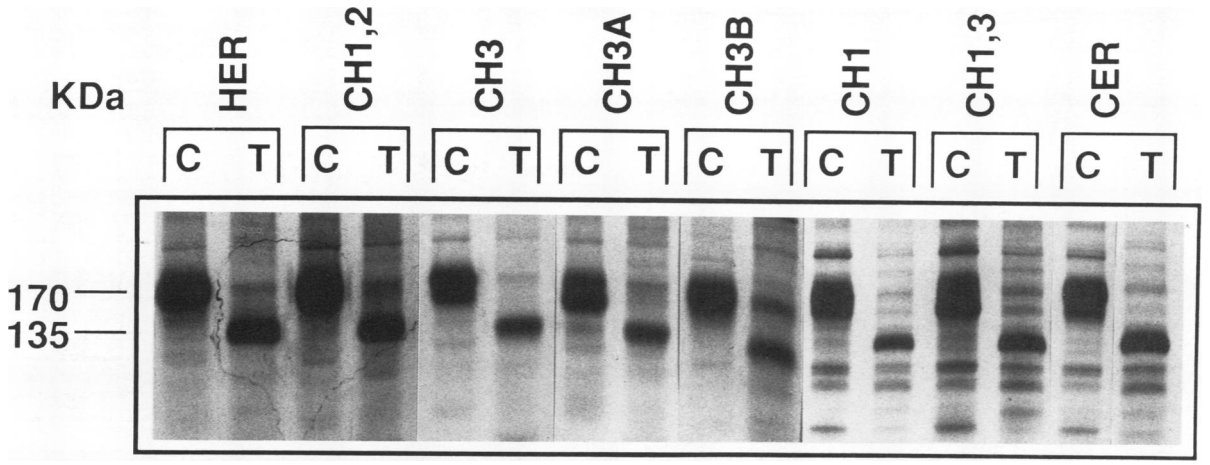

Figure 2. **Identification of expressed human, chicken, and chimeric EGFRs by immunoprecipitation of ^{35}S-menthionine-labeled cells.** Labeled cells were treated in the absence (C) or presence (T) of tunicamycin (10 μg/ml) for 12 h at 37°C and were lysed and EGFR immunoprecipitated with anti-EGFR antibodies (RK-2). The samples were analyzed by SDS-PAGE (7.5%) and by autoradiography. The autoradiograph shows the new chimeric receptors CH1, CH1,3, CH3A, and CH3B. For comparison we used the human and the chicken EGF receptors and chimeric receptors CH1,2 and CH3. Labeling of the cells with ^{35}S-methionine, immunoprecipitation of the labeled EGFR with RK-2 antibodies (Kris *et al.,* 1985), and separation by SDS-PAGE were done according to published procedure.

ble 1 summarizes the dissociation constants of ^{125}I-TGF-α to the various C/H receptor molecules. It appears that ^{125}I-TGF-α binds with a single K_d to CER, HER, and the various chimeric receptors with similar K_d values in the range of (0.32–0.56) $\times$ 10^{-9} M (Table 1). These results indicate that all the chimeric receptors retained the binding affinity of the parental molecules toward TGF-α. The exchange of domains between the human and the chicken receptors did not impair their three-dimensional structure in a way that influences their capacity to specifically bind TGF-α.

The affinity of CER toward murine EGF is ~250-fold lower than the affinity of HER toward EGF and is therefore too low to be determined by conventional binding experiments with ^{125}I-EGF (Lax *et al.,* 1988b). As such we first used a previously described displacement analysis of ^{125}I-TGF-α to determine the affinity of EGF to the chimeric receptors (Lax *et al.,* 1988b; Lax *et al.,* 1989). Figure 3 shows the displacement profiles of ^{125}I-TGF-α with increasing concentration of EGF for the various chimeric receptors. The binding affinity of CH1 toward EGF was more than twofold higher than the affinity

Table 1. Binding parameters of EGF receptor mutants for EGF and TGF-α

Cell line (receptors/cell)	K_d for EGF ($\times 10^{-9}$ M)	ΔG° kcal/mole	Δ^2G (CER)	K_d for TGF-α (M) ($\times 10^{-9}$ M)
HER ($2.5 \cdot 10^5$)	0.8 ± 0.3	12.20	3.38	0.42 ± 0.1
CER ($3.0 \cdot 10^5$)	260 ± 60	8.82		0.32 ± 0.07
CH1,2,3 ($4 \cdot 10^5$)	0.75 ± 0.2	12.23	3.41	0.56 ± 0.15
CH1,2 ($9 \cdot 10^5$)	120 ± 35	9.28	0.46	0.53 ± 0.12
CH3 ($0.7 \cdot 10^5$)	2.0 ± 0.3	11.66	2.84	0.34 ± 0.08
CH1 ($1.5 \cdot 10^5$)	110 ± 40	9.33	0.51	0.35 ± 0.07
CH1,3 ($0.6 \cdot 10^5$)	1.4 ± 0.3	11.80	3.05	0.51 ± 0.09
CH3A ($2.1 \cdot 10^5$)	40 ± 15	9.92	1.10	0.49 ± 0.12
CH3B ($3.5 \cdot 10^5$)	45 ± 20	9.85	1.03	0.31 ± 0.08

K_ds of TGF-α toward receptors were determined by directed binding experiments with ^{125}I-TGF-α followed by Scatchard (1949) analysis according to published procedures (King and Cuatrecasas, 1982). K_ds for EGF were determined from displacement curves of ^{125}I-TGF-α by EGF as described in Figure 3. The free energy of binding, ΔG°, was calculated from $\Delta G° = RTLnK = -2.3 \times 1.99 \times 293 \log K$.

Δ^2G is the difference in binding energy between the chimera and CER.

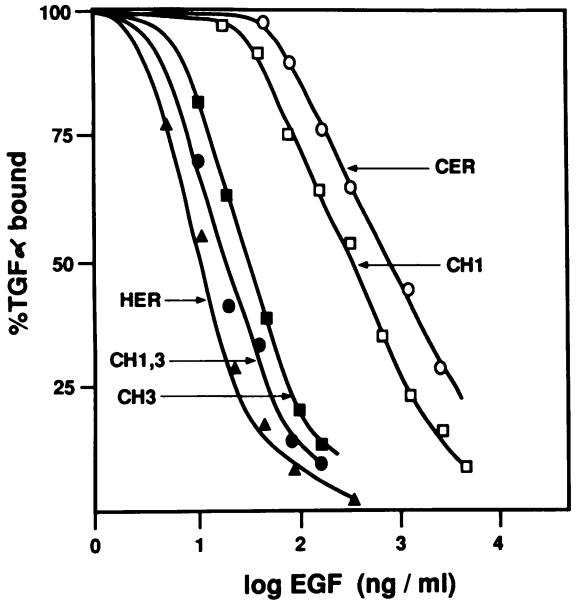

Figure 3. **Inhibition of binding of ^{125}I-labeled TGF-α by native EGF to cells expressing human, chicken, or chimeric EGFRs.** Various cell lines expressing human, chicken, or chimeric receptors were incubated with a solution containing increasing concentrations (1.4, 14, and 140 nM and 1.4 μM) of native EGF and 0.5 nM 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 cell-associated radioactivity (bound ^{125}I-TGF-α) was determined for every cell line. HER (▲); CH1,3 (●); CH3 (■); CER (○); CH1 (□).

of CER toward EGF. CH3, however, had a 130-fold higher affinity than CER toward EGF. In addition, the affinity of CH1,3 toward EGF was very similar to the affinity of HER toward EGF (Table 1). As previously indicated, domain III is a major ligand-binding region (Lax *et al.*, 1989). However, domain I also contributes to the interactions that together with domain III reconstitute high-affinity binding nearly as well as the binding of HER toward EGF.

To narrow down the region(s) of domain III that interact(s) specifically with EGF, we have introduced a unique restriction site (*Xba* I) in the middle of the cDNA encoding domain III at identical positions in both the chicken and human receptors. This allowed the replacement of either half of the chicken domain III by the corresponding regions of the human EGFR and the analysis of the contribution of each half of human domain III to EGF binding. The two chimeric receptors generated, CH3A and CH3B (Figure 1), were expressed in NIH-3T3 cells; and their EGF-binding properties are summarized in Table 1. Interestingly, both CH3A and CH3B bind EGF with similar K_ds. The K_d of EGF toward CH3A is 4×10^{-8} M, and the K_d of CH3B is $\sim 4.5 \times 10^{-8}$ M (Table 1). For comparison, the K_d of CH3 toward EGF is 2×10^{-9} M, a 20-fold higher binding affinity than the affinity of either CH3A or CH3B toward EGF. It appears that either of these regions can elevate the affinity of CER toward EGF by ~6-fold (Table 1). However, neither of these regions alone is able to fully reconstitute the high-affinity binding of CH3; therefore, both subdomain IIIA and subdomain IIIB probably contain structural determinants that together are required for the generation of high-affinity binding of EGF. The folding of domain III juxtaposes these residues to form a major part of the ligand-binding region.

All of the chimeric receptors that bind EGF with elevated affinity, including CH3, CH1,3, CH3A, and CH3B, were next analyzed by direct binding experiments with ^{125}I-EGF, and the binding data were analyzed according to the method of Scatchard (1949). It is well established that the Scatchard plots for ^{125}I-EGF binding to a variety of cells expressing either native or transduced EGFR are curvilinear (Shoyab *et al.*, 1979; King and Cuatrecasas, 1982). This is commonly interpreted as the manifestation of two receptor populations with distinct K_ds. A minor component (2–10%) represents the so-called high-affinity binding, with a K_d for EGF of $(0.1–0.5) \times 10^{-10}$ M. The majority of the receptors (90–98%) are low-affinity binding sites, with a K_d for EGF in the range of $(2–10) \times 10^{-9}$ M. Although similar K_d values were obtained for the high-affinity binding sites of HER, CH1,3, and CH3 ($K_d = [0.5–0.7] \times 10^{-10}$ M), the K_d values of their low-affinity binding sites varied: 2×10^{-9} M, 7×10^{-9} M, and 9.2×10^{-9} M, respectively (Figure 4).

The Scatchard curves of the binding of ^{125}I-EGF to either CH3A or CH3B are also curvilinear and were best fitted by a model assuming two receptor populations with two distinct K_ds. However, the high-affinity receptor population of CH3A and CH3B toward EGF has a $K_d = (0.4–0.5) \times 10^{-9}$ M. This K_d is ~10-fold higher than the K_d of the high-affinity binding of either HER, CH1,3, or CH3 toward EGF. Similarly, the low-affinity receptors of CH3A and CH3B possess an increased K_d of $(30–50) \times 10^{-9}$ M toward ^{125}I-EGF compared with the K_d of the low-affinity receptor of HER, CH1,3, and CH3.

We have also used the C/H chimera of EGFR to map the domains recognized by various monoclonal anti-EGFR antibodies (mAbs) that were generated in our own or other laboratories. By comparing the capacity of the various mAbs (Table 1) to immunoprecipitate either human or

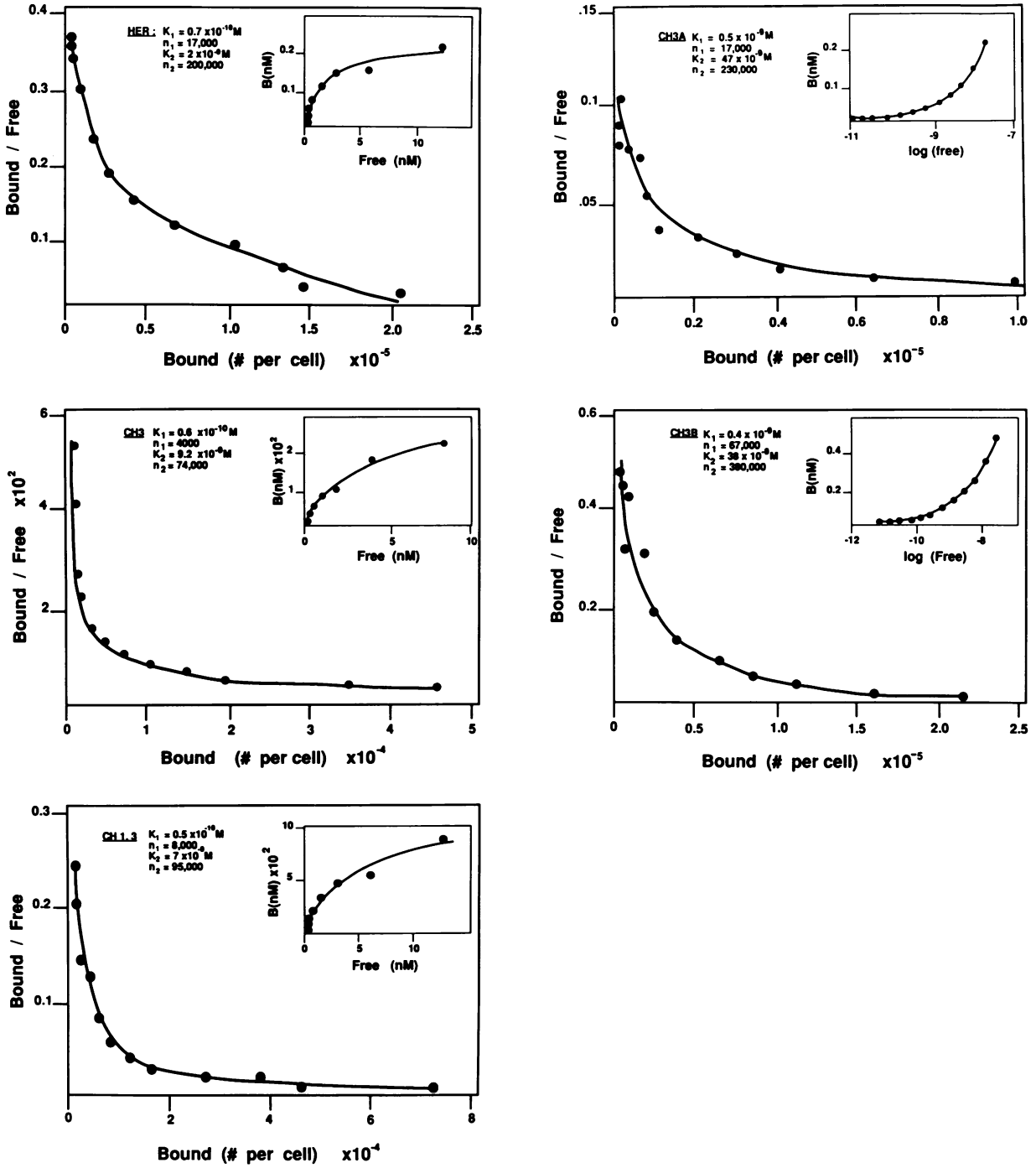

Figure 4. **Scatchard analysis of ^{125}I-EGF binding results to cells expressing human and chimeric receptors.** ^{125}I-EGF binding was determined for concentrations of ^{125}I-EGF ranging from 0.06 to 150 ng/ml for 60 min incubation at room temperature. Nonspecific binding was determined by parallel binding and data were analyzed according to the method of Scatchard (1949). Scatchard plots and binding curves (in inserts) lines expressing HER, CH3, and CH1,3 (left); and CH3A and CH3B (right) receptors. The binding affinity of EGF to cells expressing CER and CH1,2 was too low to be determined by direct binding experiments.

chicken EGFR according to published procedure (Lax *et al.,* 1989), we have concluded that all mAbs described in this report recognize the human and not the chicken EGFR (Table 1). This analysis showed that domain IIIA is recognized by mAb96 and mAbLA22 and domain IIIB is recognized by mAb425, mAb225, and mAb108 (Table 1 and Figure 5). However, mAbR1 recognizes domain II and mAb2E9 recognizes domain I. Detailed binding studies have shown that mAb425 (Murthy *et al.,* 1987), mAbLA22 (Wu *et al.,* 1989), and mAb96 (Bellot *et al.,* 1990) block both high- and low-affinity binding of ^{125}I-EGF. However, mAb108 inhibits only high-affinity binding (Bellot *et al.,* 1990) and mAb2E9 blocks only low-affinity binding of EGF (Defize *et al.,* 1989). Whereas mAb225 are also ^{125}I-EGF competitive, monoclonal antibodies mAbR1 do not influence the binding of ^{125}I-EGF to EGFR. It appears that five out of seven mAbs against EGFR recognize domain III and all of them are able to reduce the binding of ^{125}I-EGF to EGFR.

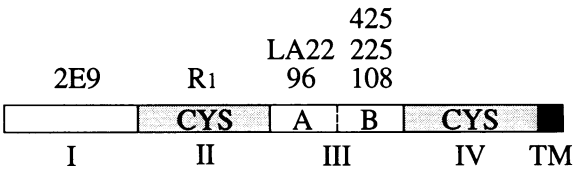

***Figure 5.* Localization of epitopes in the extracellular domain of EGFR recognized by monoclonal antibodies.** A diagram describing the extracellular ligand-binding domain of EGFR divided into four subdomains (I–IV) and the binding region of the various monoclonal antibodies analyzed in this report.

Discussion

On the basis of internal sequence homology, we had previously proposed that the extracellular domain of EGFR is composed of four subdomains, which were termed domains I–IV (Lax *et al.,* 1988b, 1990). In this study we extend the previously described chimeric C/H receptor approach to determine the contribution of domains other than domain III and to identify determinants within domain III that are important for the generation of high-affinity binding toward EGF.

TGF-α binds with similar K_ds to the chicken and human EGFR (Lax *et al.,* 1988b). Hence the values of the K_ds of TGF-α to the various chimeric receptors could provide a test for potential perturbations in the folding of the various chimeric EGFR molecules caused by the exchange of domains. Scatchard analyses of ^{125}I-TGF-α binding experiments to the various C/H chimeric receptors, to CER, and to HER indicate that all these receptors have a similar affinity toward TGF-α with K_ds in the range of (0.32–0.56) $\times$ 10^{-9} M. This result shows that the exchange of domains between human and chicken receptors did not perturb the overall structure of the C/H chimera, further supporting the domain hypothesis for the structure of the EGFR (Lax *et al.,* 1989, 1990).

Using three different experimental approaches, we had previously concluded that domain III serves as a major ligand-binding domain for EGF. First, CH3 chimera binds EGF with ~150-fold higher affinity than chicken receptor and with only 2- to 3-fold lower affinity than human EGFR (Lax *et al.,* 1988a). Second, domain III is contained within the ^{125}I-EGF affinity-labeled fragment generated by CNBr cleavage (Lax *et al.,* 1988a), and, finally, deletion mutants lacking domain I bind EGF with 10-fold reduced affinity compared with the binding of intact EGFR (Lax *et al.,* 1990).

Because the analysis of chimeric C/H receptors provides a functional approach for the localization of the ligand-binding region, we have extended this approach by generating additional C/H chimera to narrow down the region(s) in domain III as well as to reveal additional regions in the extracellular domain that contribute to high-affinity binding. For this purpose, we have generated chimera CH3A, which contains the 5′ half of human domain III introduced in a chicken EGFR background, and chimera CH3B, which contains the 3′ half of human domain III introduced to the chicken receptor. Analyses of ^{125}I-EGF binding experiments indicated that both chimeric receptors of CH3A and CH3B have similar high- and low-affinity binding toward EGF, indicating that both halves of domain III interact specifically, albeit weakly, with EGF.

It appears, therefore, that domain III of EGF receptor contains at least two noncontiguous regions that together provide most of the interactions that define binding specificity of EGF to the receptor. Direct binding experiments with ^{125}I-EGF to either CH3A or CH3B produce curvilinear Scatchard plots which are best fitted by a model assuming two receptor populations with distinct K_ds. Moreover, as with the native EGF-receptor, treatment with phorbol ester (PMA) abolished the high-affinity binding for ^{125}I-EGF of either CH3A or CH3B mutants (data not shown). Similar results were also obtained with CH3 and CH1,3 (data not shown). The molecular nature of the high- and low-affinity binding sites of normal or wild-type EGFR is not fully understood. We had previously proposed and ob-

tained recent evidence that the high-affinity binding of EGF is by a dimeric receptor possessing higher intrinsic ligand-binding affinity (Boni-Schnetzler and Pilch, 1987; Yarden and Schlessinger, 1987a,b; Schlessinger, 1988; Bellot *et al.*, 1990). If this is the case, then CH3A and CH3B should oligomerize in response to EGF. Additionally, domain III may be also involved in receptor-receptor interactions stabilized by ligand binding.

Studies using monoclonal anti-EGFR antibodies provide additional clues as to the role of domain III in ligand binding. Five out of seven mAbs against EGFR analyzed in this study bind to domain III. mAb96 (Bellot *et al.*, 1990) and mAbLA22 (Wu *et al.*, 1989), which block both high- and low-affinity ^{125}I-EGF binding to EGFR, bind to subdomain IIIA (Table 1). mAb425 (Murthy *et al.*, 1987) binds to domain IIIB and blocks both high- and low-affinity binding of ^{125}I-EGF to EGFR. mAb108, which specifically blocks the high-affinity binding of EGF (Bellot *et al.*, 1990), binds to subdomain IIIB. mAb225, which binds to domain IIIB, clearly also reduces ^{125}I-EGF binding to EGFR (Kawamoto *et al.*, 1983). Interestingly, mAb2E9, which binds to domain I, blocks only low-affinity binding of ^{125}I-EGF (Defize *et al.*, 1989). However, mAbR1 binds to domain II and does not influence the binding of ^{125}I-EGF to EGFR. The fact that mAb2E9, which binds to domain I, is able to block only low-affinity EGF binding provides further support for the notion that domain I is also involved in ligand-binding recognition.

The binding parameters of various chimera may be better understood by calculating the contribution of each human domain to the free energy of EGF binding ($\Delta G°$) to the chicken EGFR, as calculated from the equilibrium constants (Table 2). The increase in binding energy of EGF by HER versus CER is 3.38 kcal/mole. Each half of domain III contributes >1 kcal to CER, and the entire CH3 contributes 2.84 kcal to CER binding. This suggests that the contact residues are not localized and are probably spread over domain III and that the folding of this domain brings them together to form the ligand-binding site. This folding brings about an additional 0.62 kcal on top of the additive contributions of subdomains IIIA and IIIB alone. Domain I contributes only 0.51 kcal to the binding of CER, but domains III and I together are able to additively reconstitute in CER almost the entire energy of binding of HER toward EGF (3.3 kcal) (Table 2). The results therefore suggest that most of the residues that are in contact with EGF reside in domain III. The contribution of domain I to the binding energy may be due to either some minor interactions between EGF and domain I or to domain-domain interactions between domains I and III, which may reflect conformational changes induced on EGF binding. Such interactions are common in allosteric proteins where ligand binding alters the interaction between neighboring subunits, thus bringing about an allosteric change. It is also possible that the binding properties toward EGF are determined by one long region spanning across domain IIIA and IIIB of EGFR, the interruptions of which destroy some critical determinants. We had previously proposed a four-domain model for the extracellular portion of EGFR in which the ligand-binding region lies in a cleft between domains III and I (Lax *et al.*, 1990). Studies on the structure of the extracellular portion of the EGFR will test the validity of this prediction.

Table 2. Recognition of various chicken/human EGFR chimera by different monoclonal antibodies

	EGFR						
mAb	HER	CER	CH1	CH1,2	CH3	CH3A	CH3B
108	+	−	−	−	+	−	+
96	+	−	−	−	+	+	−
R1	+	−	−	+	−	−	−
LA22	+	−	−	−	+	+	−
2E9	+	−	+	+	−	−	−
225	+	−	−	−	+	−	+
425	+	−	−	−	+	−	+

Interaction of mAbs with various EGFR mutants was analyzed by the use of immunoprecipitation experiments that were described in detail in an earlier publication (Lax *et al.*, 1988). R1 was purchased from Amersham, LA22 was obtained from D. Sato, 2E9 was obtained from Z. de Laat, 225 was obtained from J. Mendelsohn, and 425 was obtained from M. Das.

Materials and methods

Preparation of constructs

The construction of the C/H chimeric receptors CH1,2,3, CH1,2, and CH3 was previously described (Lax *et al.*, 1989). New C/H chimera were prepared by introducing unique restriction sites in the previously mutated cDNA of human and chicken EGFRs (denoted HERM and CERM in Lax *et al.*, 1989), which contained the *Sna*BI and *Mlu* I sites at the end of domain II and the beginning of domain IV, respectively (Lax *et al.*, 1989). A unique *Xba* I site was introduced in the middle of domain III at position 1445 and 1568 of HER and CER, respectively (Ullrich *et al.*, 1984; Lax *et al.*, 1988b). The oligonucleotides CCTTTGAGAATCTAGAAATC and AATCTAGAGATTATC were used to mutate HERM and CERM, respectively, and the mutated nucleotides are underlined. The mutation did not change any amino acid and

subdivided domain III to almost two equal portions (Xba cuts 5′ to the codon of Leu 399 and 400 of HER and CER, respectively). These constructs were denoted HERMX and CERMX. Chimera CH3A, containing the 5′ half of domain III of HER, was prepared by ligating the *Sna*BI-*Xba* I fragment from HERMX into CERMX, from which the *Sna*BI-*Xba* I fragment was removed by agarose electrophoresis. Chimera CH3B containing the 3′ half of domain III of HER was prepared by ligating the *Xba* I-*Mlu* I fragment from HERMX with CERMX, from which the *Xba* I-*Mlu* I fragment was removed by gel electrophoresis.

To construct chicken chimera with domain I of human EGFR, we introduced an *Sph* I site (GCATGC) that generates Ala-Cys codons in a position involving the first Cys codon of domain II (Cys 163 and 164 of HER and CER, respectively). The mutation substituted Leu for Ala at codon 162 or 163 of HER and CER, respectively. The oligonucleotides CACCTGGGCGCATGCCAAAAGTCGT and AATCTTTCTGCATGCCCAAAATGC were used to mutate HERM and CERM (Lax *et al.*, 1989) and were denoted HERMS and CERMS, respectively. Chimera CH1 was prepared by ligating the *Sph* I fragment of the M13 RF from HERMS with CERMS, from which the same fragment was removed by agarose electrophoresis. The *Xho* I fragment containing the entire insert was isolated and ligated with pLSV (Lax *et al.*, 1989). To generate chimera CH1,3, we isolated the *Sna*BI-*Mlu* I fragment from CH3 and inserted it into CH1, from which the same fragment was removed. The mutant plasmids were prepared by the CsCl method and used to transfect NIH-3T3 cells. Mutagenesis was performed on M13 um 20 (IBI) single strand DNA by the use of the Amersham (Arlington Heights, IL) kit for site-directed mutagenesis according to the manufacturer's instructions. The mutations were verified by DNA sequencing. The mutant receptor DNA was isolated from the M13 RF and ligated into PLSV as described (Livneh *et al.*, 1986; Lax *et al.*, 1988–1990).

Transfection and cell culture

NIH-3T3 cells (clone 2.2) devoid of endogenous EGFR were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY) with 10% fetal bovine serum. Cells growing in 10-cm dishes were cotransfected with a mixture (30 μg: 1 μg per dish) of expression vectors containing mutant cDNA together with pSV2Neo 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 per 10-cm dish, and grown in the presence of 0.8 mg of Geneticin G418 (GIBCO) per ml in the medium for neomycin resistance selection. Resistant clones were picked after 3 wk and tested for expression of the mutant receptors by immunoprecipitation with anti-EGFR antibody RK2 (Kris *et al.*, 1985) and receptor self-phosphorylation. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Biosynthetic labeling

Subconfluent cells in 10-cm dishes were washed with methionine- and cysteine-free DMEM and grown for 12 h in methionine- and cysteine-free DMEM supplemented with 10% fetal calf serum (FCS) containing 50 μCi/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 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA]; 1 μg/ml aprotinin; 1 μg/ml leupeptin; and 1 mM phenylmethylsulphonyl fluoride [PMSF]), incubated for 30 min on ice, and spun for 10 min in an Eppendorf centrifuge in the cold. Three micrograms of protein A-Sepharose per sample were suspended in 20 mM HEPES (pH 7.5), washed with 20 mM HEPES, and incubated for 30 min at room temperature with anti-EGFR 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 mM NaCl, and 5 mM EGTA; once with HNTG buffer; 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(hydroxymethyl) aminomethane (Tris)-HC1 (pH 8.0) and 0.1% Triton X-100. The 3 vol 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 with ^{125}I-EGF and ^{125}I-TGF-α

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 and were allowed to grow for 48 h to confluency in DMEM containing 10% FCS. Mouse EGF (Toyobo, Tokyo) or human TGF-α (Bachem, Torrance, CA) were iodinated by the use of the chloramine-T method to a specific activity of 100 000–200 000 cpm/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. Nonspecific binding was determined by parallel binding experiments to parental cells lacking endogenous EGFRs (Lax *et al.*, 1988b, 1989). 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. Similar results were obtained when the binding experiments were performed at 4°C (data not shown). The cells were lysed in 0.5 ml of 0.5 M NaOH for 30 min at 37°C, and their radioactivity was measured by the use of a gamma counter to determine the amount of ligand bound to the cell surface. In displacement experiments, 0.5 nM of ^{125}I-TGF-α was displaced by 0.1–5000 ng/ml of native EGF.

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